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THE BRITISH JOURNAL
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EXPERIMENTAL PATHOLOGY

THE NATURE AND SYSTEMATIC POSITION OF *B. PARATYPHOSUS C.*

F. W. ANDREWES AND SHEFFIELD NEAVE.*
From the Laboratories of St. Bartholomew's Hospital, London.

Received for publication May 4th, 1921.

DURING the war there were a number of outbreaks of infective disease in Eastern Europe and in Asia which were traced to a bacillus of the paratyphoid group apparently distinct from *B. paratyphosus A* and *B*, though having some affinities with the latter. The name *B. paratyphosus C* has been employed for this organism, and the evidence connecting it with the disease is of the same validity as in the case of the other paratyphoids—that is to say, the organism is readily recoverable by blood culture in those infected and it is present after death in the spleen or other viscera, while the serum of the patient exhibits characteristic agglutination of the organism. The symptoms produced by infection have been varied. At times they have resembled a paratyphoid fever of short course, in other cases they have been those of acute gastro-enteritis or dysentery, in others again the phenomena have been those of an acute septic condition, while in some they have been chiefly pulmonary, taking the form of bronchitis and pneumonia. Intestinal ulceration has been sometimes present, but often absent, and the bacillus has seldom been cultivated from the stools, though not infrequently from the urine. The mortality has been high in some outbreaks, low in others. Two non-fatal cases are known to have occurred in England since the war: one, in a little girl at St. Thomas's Hospital in 1920, has been published by Dudgeon(1); the other, in a man returned from Palestine, occurred at St. Bartholomew's Hospital in 1919 and is described in this paper. It was the starting-point of the investigation into the nature of the organism the results of which are here set forth.

The patient, a clerk, aged 25 years, served from March, 1917, to February, 1919, in the Royal Garrison Artillery in Palestine, and in no other field of the war. In April, 1918, he had an attack of amœbic dysentery which yielded to

* Owing to Dr. Neave's absence from this country I have had to undertake the writing of this paper, and must assume responsibility for its deficiencies. Dr. Neave has helped me in the work throughout and his name should be associated with it.—F. W. A.

emetin. Later in the same year he was for one week in hospital in Alexandria with unexplained pyrexia, but quickly recovered and rejoined his unit in October, 1918. From that time till his return home he remained well. He left Palestine in February, 1919, in a transport crowded with soldiers returning from Mesopotamia. On reaching England in March he was perfectly well, and after two weeks' furlough resumed his work as a clerk in the London General Omnibus Company.

On May 3rd he had an illness diagnosed as influenza, and three days later this was complicated by pneumonia. He began to get up on May 15th, but had a rigor on May 18th, and on May 21st had diarrhœa lasting three days. He remained somewhat febrile, with occasional rigors, and on June 9th was admitted to St. Bartholomew's Hospital under the care of Dr. Drysdale. He was very ill, with irregular fever and rigors; there were no abdominal symptoms, but obscure signs at the base of one lung, which ultimately proved due to a small pleural effusion. Blood culture on June 23rd yielded in two tubes out of six a bacillus culturally resembling *B. paratyphosus B*, but inagglutinable with paratyphoid B serum. A culture sent to the Lister Institute was at once identified by Dr. Schütze as identical with the Bagdad strains of paratyphoid C. On June 30th the agglutinating powers of the patient's blood were investigated, using Dreyer's method; he had been inoculated with T.A.B. vaccine rather less than a year previously. The result was as follows:

B. typhosus 1 : 400 = 32 Dreyer units.
B. paratyphosus A, 1 : 164 = 39 Dreyer units.
B. paratyphosus B, 1 : 656 = 187 Dreyer units.
B. Aertrycke (Newport), 1 : 267.
„ (Mutton), 1 : 300.
Gärtner's bacillus, 0.
Patient's own bacillus, 1 : 1600.

A week later the titre to the patient's own bacillus had fallen to 1 in 1000, and that for T. A. and B. was practically unchanged, B showing a slight fall from 187 to 178 units. The fæces were negative on bacteriolcgical examination. On July 10th the urine was examined: it contained a moderate amount of pus and yielded an abundant and pure growth of a bacillus identical with that obtained from the blood, except that it was agglutinated by paratyphoid B serum to about half titre. The patient was put on urotropin and the pus and bacilli disappeared from the urine in a few days.

The man recovered; the fever ceased about the end of June; there was some femoral thrombosis early in July. After he left the hospital he had an attack of appendicitis, but when seen again in November he was quite well, and his urine was normal and negative on culture.

HISTORICAL.

The term "*B. paratyphosus* C" was proposed in 1908 by Uhlenhuth and Hübener[2] for a race of bacilli encountered by them in pigs in the course of their investigations on swine fever. They stated that it was culturally indistinguishable from *B. suipestifer*, but inagglutinable with sera of the hog cholera

group and by Gärtner serum, while its own serum was equally without influence on hog cholera bacilli. They found the organism in the organs of pigs suffering from swine fever, in the stools of healthy men, pigs and calves, and in sausages, and they further identified it with a certain group of the organisms concerned in calf-dysentery. In connection with these statements it must be remembered that the Uhlenhuth school draws no sharp distinction between *B. suipestifer* and *B. paratyphosus B*, and regards the Aertrycke strains as practically identical with the latter organism; further, it places little reliance on the absorption test in serological work. There is, nevertheless, some evidence in the work of Dienes and Wagner, to be mentioned below, to suggest the identity of one, at least, of Uhlenhuth's strains with the organism we are about to consider. Outbreaks of disease due to members of the paratyphoid group inagglutinable with ordinary sera have since been observed from time to time in Germany. Heimann,(3) for example, has described an outbreak of meat-poisoning at Hildesheim in 1911, due to pork, in which the bacillus was of this type, but as he did not employ for his fermentation tests the substances now known to be of service in distinguishing between paratyphoid B and C and suipestifer, and as he did not test his bacillus against a suipestifer serum, it is impossible to be sure of the nature of the organism.

In 1914, or earlier, at Peshawur in India, a bacillus was isolated which Schütze(4) has tested and identified with *B. paratyphosus C.*

In 1915 Neukirch,(5) attached to the Turkish army in Armenia, encountered a considerable outbreak of disease due to an organism which he called the "Erzindjan bacillus." Cultures sent to Germany were there identified by means of "Voldagsen" and "Glässer" sera (of which more anon) with the suipestifer group.

In 1916 the same organism was observed in several of the fields of war. Weil and Saxl(6) describe three cases in Russian prisoners in Volhynia, and Weil(7) later had two further cases in Albania. Weil compared his bacillus with that of Neukirch and found it identical. Dienes and Wagner,(8) at Lemberg, investigated a large number of cases, mostly in Russian prisoners, and found bacilli agglutinating with "Voldagsen" serum, as well as a race apparently differing from *B. paratyphosus C.* They applied agglutination and absorption tests, and, happening to have a strain of Uhlenhuth's paratyphoid C from Kral's laboratory, they found that this absorbed the specific agglutinin from the sera made with their own strains. This is an important piece of evidence connecting the recent war strains with Uhlenhuth's organism. Dienes and Wagner affirm the identity of their bacilli of Group I with Weil's Volhynian strains and Neukirch's Erzindjan bacillus as well as with Uhlenhuth's paratyphoid C.

Meanwhile Hirschfeld,(9) bacteriologist to the Serbian army, had met with a disease resembling enteric, but due to a special bacillus which he called *Paratyphoid C.* He added this organism to the protective vaccine he was using. Dudgeon(1) also, in Macedonia, was in communication with Hirschfeld and observed cases of his own. He has done experimental work with the bacillus, which seems quite identical with that found by the other observers named.

Quite independently British workers in Mesopotamia met with the same bacillus in cases at Bagdad. These have been described by Mackie and

Bowen[10] and by MacAdam,[11] and cultures of these strains were brought back to England by Ledingham. Garrow[12] has also reported a case of paratyphoid C from East Africa.

These various findings may be regarded as establishing a type of paratyphoid infection of variable clinical character, due to a bacillus distinct from *B. paratyphosus A* and *B*, but having a certain serological relation with the latter. Many observers have found that while some paratyphoid C strains fail to react at all with a paratyphoid B serum, others are agglutinated by it to a quarter or a half of its full titre; it has been observed also that a strain may change in this respect while under cultivation, a bacillus, at first incapable of agglutinating with paratyphoid B serum, gradually aquiring that property; Hirschfeld reports a change in the opposite direction. More than this, it was found by Neukirch that when the bacillus was repeatedly cultivated from a single case, sometimes it reacted to some degree with paratyphoid B serum and sometimes not. Its agglutination with its own serum does not appear to vary.

CHARACTERS OF *B. VOLDAGSEN* AND *B. TYPHI SUIS* (GLÄSSER).

In the course of our study of the paratyphoid C bacillus we have compared it with a wide range of members of the Salmonella group, employing not only agglutination, but the absorption test. The organisms with which we have worked have been as follows:

B. typhosus, several strains; *B. paratyphosus A*, several strains; *B. paratyphosus B*, 8 or 10 strains; *B. Aertrycke* (Newport race), 2 strains; *B. Aertrycke* (Mutton race), several strains; *B. paratyphosus* C, Hirschfeld's Serbian strain and 2 from Bagdad; *B. Gärtner*, several strains; *B. suipestifer*, 8 or more strains, chiefly American and German; *B. Voldagsen*; *B. typhi suis* of Glässer, 2 strains.

We owe many thanks to the various workers who have supplied us with these strains; above all to the Lister Institute and to Prof. Jensen of Copenhagen, who kindly sent a supply of German strains, chiefly from Uhlenhuth's laboratory. We made our own sera from rabbits, but some were sent us from elsewhere. The following sera were employed:

B. typhosus, B. paratyphosus A, B. Gärtner. B. paratyphosus B: 2 of our own make and 1 from Oxford. *B. Aertrycke:* Newport and Mutton strains. *B. paratyphosus C:* 3 sera, viz. from the Hirschfeld strain and from the blood and urine strains of our own case. *B. suipestifer*: 3 sera, made respectively from English, American and German strains. *B. Voldagsen* and *B. Glässer.*

Inasmuch as *B. Voldagsen* and *B. typhi suis* (Glässer) played a part in the identification of *B. paratyphosus C* by the German observers, and as these organisms have been little if at all studied in England, it may be well to state some facts about them.

B. suipestifer, Voldagsen, was described by Dammann and Stedefeder [13] in 1910 as the cause of a disease in young pigs resembling swine-fever, and known as "Ferkel typhus" or "sucking-pig typhoid." Considerable dispute has arisen about it in Germany: the Uhlenhuth school maintain that it is a secondary

invader, and that the primary disease is due to the usual filter-passer of swine-fever—a position denied by Pfeiler. These disputes need not concern us here; the interest lies in the fact that the Voldagsen bacillus differs considerably from *B. suipestifer* in its cultural characters, while evidently allied serologically.

B. typhi suis was described by Glässer ([14]) as similarly a cause of disease in pigs, and is by many regarded as identical with the Voldagsen bacillus. Like that organism it differs culturally from *B. suipestifer*, but resembles it in serological characters.

By the kindness of Prof. Jensen we received a culture of *B. Voldagsen*, of German origin, and two Glässer cultures, one emanating from Prof. Pfeiler's laboratory at Bromberg and one from Prof. Poppe, of Berlin. When these strains were plated out on agar they were seen to be much less vigorous in their growth than ordinary suipestifer strains; the colonies of *B. Voldagsen* in particular were minute, irregular in shape and very transparent, the Glässer colonies more regular and a little larger. Both organisms were actively motile and longer and thinner than *B. suipestifer*. The fermentative reactions were almost the same in the two types: no gas was formed in any sugar, or only very minute traces, except that one of the Glässer strains gave gas in xylose. Mannite was scarcely attacked; one Glässer strain would not ferment it at all, the other took 13 days and the Voldagsen strain 15 days to produce acid. The Glässer strains were similarly shy in attacking dulcite, taking 15 and 19 days to produce acid; the Voldagsen bacillus took only 4 days. All three strains, unlike *B. suipestifer*, readily attacked arabinose. Instead of producing an alkaline reaction in litmus milk, that fluid, after an initial acidity, became neutral again; even after 46 days there was no alkaline reaction, except for a faint trace in the case of *B. Voldagsen*. It will be seen that these characters differ so widely from those of *B. suipestifer* and the paratyphoids that one would be inclined to reckon the Voldagsen and Glässer bacilli as specifically distinct.

We prepared rabbit sera from the Voldagsen strain (titre about 50,000) and from one of the Glässer strains (titre about 20,000), and in numerous experiments we tested the agglutinating powers of these sera upon other Salmonellas, and the effect of our other Salmonella sera upon the Voldagsen and Glässer strains. Formalinised broth cultures of standard opacity were used by the macroscopic method, the tubes being partially immersed for two hours in a water-bath at 54° C. The Voldagsen serum agglutinated ordinary suipestifers to its full titre, and paratyphoid C strains to about half titre: paratyphoid B strains and Aertryckes were only weakly agglutinated and the Glässer strains scarcely at all. The Glässer serum agglutinated paratyphoid C strains and some suipestifers to titre or nearly so, the Voldagsen bacillus only weakly and the paratyphoid B strains and Aertryckes scarcely at all. Somewhat similar relations were found when Voldagsen and Glässer strains were exposed to the action of the various other sera. It was apparent that the Voldagsen and Glässer strains were by no means serologically identical, and this opinion was confirmed by absorption tests, in which it was found that a Voldagsen serum was completely exhausted of all agglutinins by saturation with the Glässer bacillus, while the specific agglutinin in the Glässer serum was almost unaffected by saturation with the Voldagsen bacillus.

It is thus manifest that in the suipestifer group serological and cultural characters do not necessarily go hand in hand.

One further remark may be made here, though it is not germane to the subject of the paratyphoid C bacillus. Owing chiefly to the work of Bainbridge ([15]), it has become customary in this country to identify the Aertrycke bacillus with *B. suipestifer*. While it is clear from Bainbridge's careful work that the strains of Aertrycke and suipestifer which he used were serologically identical, we are inclined to believe that the strains of suipestifer supplied to him from Germany, where they use the term more loosely than we do, were not identical with the true hog cholera bacillus of Salmon and Smith. It is more likely that his suipestifer was in error than his Aertrycke strain, for we have obtained from Prof. Hutchens, of Newcastle, a culture believed to have descended from the strain of Aertrycke Bainbridge used, and this, though now poorly agglutinable, can still definitely be identified with the "Mutton" type of Aertrycke. Our own observations, extending over a considerable range of Aertryckes and suipestifers, indicate constant cultural and serological differences which forbid their identification. The Aertrycke strains are very much more closely allied to *B. paratyphosus B* than to *B. suipestifer*, though they appear to contain a trace of suipestifer antigen absent in *B. paratyphosus B*.

THE CULTURAL AND FERMENTATIVE CHARACTERS OF *B. PARATYPHOSUS C* COMPARED WITH THOSE OF OTHER SALMONELLAS.

All the members of the group agree in being motile bacilli, negative to Gram's stain, not liquefying gelatin and forming no indol. All fail to ferment lactose and saccharose: there are, however, four sugars or allied substances which enable us to effect a certain amount of differentiation amongst them; they are xylose, arabinose, dulcite and inosite. We have tested the reactions of seventy or more strains of known serological properties and our results confirm those of other observers. They are shown in the following table:

	Xylose.	Arabinose.	Dulcite.	Inosite.
B. paratyphosus A	−	+	+	−
„ *B*	+	+	+	+
„ *C*	+	+	+	−
B. Aertrycke (Newport)	+	+	+	−
„ (Mutton)	+	+	+	+
B. Gärtner	+	+	+	−
B. columbensis	+	+	+	−
B. suipestifer	+	−	−	−

The only exceptions we have found to the reactions shown in this table were in one strain out of eight of the Mutton type of Aertrycke and one out of twelve paratyphoid B's tested which failed to ferment inosite, and in three strains of suipestifer, two of which gave a late and imperfect reaction with dulcite and the other an initial transient acidity in arabinose. Dulcite and arabinose fermenting strains of *B. suipestifer* are known to occur or to arise by mutation, but the bulk of our strains were constant in refusing to attack these substances, and we failed to produce an arabinose fermenting mutant when two single

bacillus strains were cultivated for several generations on arabinose agar plates.

It is well known that the various Salmonella strains mentioned above speedily produce an alkaline reaction in litmus milk; only *B. paratyphosus A* fails to do this, the alkaline reaction being delayed for several weeks. *B. paratyphosus C* forms alkali rather slowly. We adopted also another method of determining the rate of alkali formation in this group and its allies, testing some seventy strains. An ovo-mucin medium was tinted with phenol red and its reaction was so adjusted that after the final sterilisation it was P_H 6. The tubes were inoculated with one loopful of a 24-hour broth culture of each strain tested, and compared from day to day with a standard series of phenol red tubes ranging from P_H 6·6 to P_H 8. The time taken by the inoculated tubes to reach P_H 8 varied somewhat even with the same race; the figures obtained for this limit were as follows:

B. coli communis, 2 days; *B. columbensis*, 2 days; *B. Gärtner*, 3 to 6 days; *B. paratyphosus B*, 3, but sometimes 6 days; *B. Aertrycke*, 3 up to 6 days; *B. suipestifer*, 3 to 8 days; *B. paratyphosus C*, 8 to 10 days; *B. typhosus*, 8 to 13 days; *B. Voldagsen* and *B. Glässer*, 10 to 13 days; *B. paratyphosus A*, 13 days.

The figure for *B. fæcalis alkaligenes* could not be determined because it would not grow at P_H 6.

The various races were also roughly tested as regards production of hydrogen sulphide by growing them in broth tubes with basic lead acetate. *B. typhosus*, *B. paratyphosus A*, and *B. columbensis* gave practically no blackening, and there was not much in the case of *B. Voldagsen* and the Glässer strains; the remainder formed H_2S readily, the paratyphoid C's equally with the rest. Like most other Salmonellas the paratyphoid C's reduce neutral red, with fluorescence.

B. paratyphosus C is thus seen to be distinguished from *B. paratyphosus B* and the Mutton type of *B. Aertrycke* by its inability to ferment inosite and its slower rate of alkali production. It is culturally indistinguishable from Gärtner's bacillus and the Newport type of *B. Aertrycke*, except by its slower alkali formation. It differs from *B. suipestifer* in readily fermenting dulcite and arabinose. These are perhaps not sufficiently weighty reasons for regarding it as a distinct race if it were not for its peculiar serological characters, to which we will now turn.

THE SEROLOGICAL CHARACTERS OF *B. PARATYPHOSUS C.*

The inquiry upon which we have been engaged has extended over the various races of the Salmonellas pathogenic for man, and cannot fully be discussed here; but the relations of *B. paratyphosus C* to *B. suipestifer* render it necessary to give a short account of our observations on the latter organism in its serological aspects, limiting ourselves to the property of agglutination.

The puzzling relationships of the Salmonella group become a little clearer when search is made for particular antigenic components. The criterion of a specific antigen, *quâ* agglutination, is that it reacts only with the specific agglutinin which it evokes, and that this specific agglutinin cannot be absorbed

by other antigens. We fully recognise that the doctrine of multiple antigenic components is no more than a working hypothesis, but it is necessary to postulate something of the sort if we are to offer any sort of explanation of the phenomena observed.

We have to consider three antigenic components in discussing *B. paratyphosus C.* Two of them belong essentially to *B. suipestifer* and the third to *B. paratyphosus B.* The reasons for assuming two suipestifer antigens are as follows: We have worked with nine suipestifer strains of porcine origin, apart from the Voldagsen and Glässer races. Now when these strains are submitted to agglutination by a Glässer serum, or the serum which we made from the *blood* strain of paratyphoid C from our own case, they are sharply differentiated into two groups. Either they are agglutinated to the full titre of the serum, or only to a weak degree (3 to 10 per cent. of the full titre).

Group I (agglutinated to full titre by the sera named above).

Swine fever (Royal Veterinary College); hog cholera (Arkansas); type suipestifer (No. 42), (Indiana); *B. typhi suis* (Glässer), (both our strains); paratyphoid C (all strains tested).

Group II (agglutinated very weakly by the sera named above).

B. suipestifer (Künzendorf strain); *B. suipestifer* (strains 327, 338, 339), (Uhlenhuth); *B. suipestifer* N_2 (isolated at the Lister Institute by Schütze); " intermediate 202 " (Indiana); *B. Voldagsen.*

This differentiation is not sharply effected by any of the other Group I sera we have employed, not even by that of the urine strain of paratyphoid C from our case. Sera of Group II—*e. g.* a Voldagsen or suipestifer Künzendorf serum—agglutinate Groups I and II almost equally, except that they bring down the paratyphoid C's only to half titre, and the Glässer strains hardly at all.

The absorption test emphasises the distinction between the two groups of *B. suipestifer.* A member of either group will completely, or almost completely, remove all agglutinins from a serum belonging to its own group. But whereas a member of Group I will exhaust a Group II serum, a member of Group II fails to produce much effect in reducing the titre of a Group I serum, except for members of Group II. The following example of reciprocal absorptions between *B. Voldagsen* (Group II) and *B. Glässer* (Group I) will serve to illustrate this:

A. *Voldagsen Serum absorbed with* B. Glässer (*dose* $5\frac{1}{2}$ *billion bacilli per c.c. undiluted serum*).

Strains used as test.	Titre of unabsorbed control.	Titre after absorption.
Aertrycke: Newport	7,700	0 (at 1 in 125).
Paratyphoid B	2,300	0
Paratyphoid C (Hirschfeld)	40,000	0
Hog cholera (Arkansas)	66,000	0
Suipestifer (Künzendorf)	68,000	0
B. Voldagsen	70,000	0
B. Glässer	200	0

B. *Glässer Serum absorbed with* B. Voldagsen (*dose* 10 *billion per c.c. serum*).

Strains used as test.	Titre of unabsorbed control.	Titre after absorption.
Paratyphoid C (Hirschfeld)	17,000	15,000
Hog cholera (Arkansas)	10,000	10,000
B. Glässer	17,000	17,000
Suipestifer (Künzendorf)	1,175	0 (at 1 in 125).
Suipestifer 339 (Uhlenhuth)	1,525	0
B. Voldagsen	1,100	0

It is not a little remarkable that these two races, showing similar cultural differences from other suipestifers, should exhibit the serological dissimilarities which divide the suipestifers themselves into two groups. It is also remarkable that the Glässer bacillus, though agglutinated to such a trifling degree by the Voldagsen serum, nevertheless effects such complete absorption.

The only explanation which we can frame for these facts is to imagine two suipestifer antigens, which we may call 1 and 2, and to suppose that Group I contains both these, while Group II contains only one.

The antigenic structure of the suipestifers is further complicated by the intrusion of a paratyphoid B element to a variable extent in different races. The evidence for this is derived both from agglutination and absorption. *B. Glässer* alone seems quite free from this element, and we believe that *B. paratyphosus* B (at least in its human type) is equally free from any suipestifer element. A paratyphoid B serum will not agglutinate *B. Glässer* at all, nor will a Glässer serum agglutinate *B. paratyphosus B*: no absorption of the specific agglutinin is witnessed in either serum on saturation with the other bacillus, however large the dose employed. One of our paratyphoid C strains, that isolated from the blood of our own case, was also, at first, almost equally free from the paratyphoid B element, its serum behaving like a Glässer serum; but under cultivation the paratyphoid B element gradually asserted itself, as we shall show later. The strain isolated from the urine of the same case contained from the first a large paratyphoid B element. In other suipestifers and in *B. Voldagsen* the paratyphoid B antigen is present in varying degree, as testified by slight or moderate mutual agglutination and absorption.

Turning now from this digression as to the grouping of the *B. suipestifer* strains to our observations on *B. paratyphosus C* itself, we will deal only with our own strains, isolated respectively from the blood and urine of the case described above, and with the Serbian Army strain from Hirschfeld, which we have used for purposes of comparison. We will speak of these as the Witts' blood strain, the Witts' urine strain and the Hirschfeld strain.

So far as their relations *inter se* are concerned, these three strains appeared identical. Apart from the fact that the Witts' blood strain was uniformly less agglutinable with all sera (by some 50 per cent.) than the other two, it may be said that the serum of any one of the three agglutinated the other two to full titre. Further, any one of the three completely exhausted the sera of the other two of primary and secondary agglutinins alike. A single example of this will suffice:

Serum: Witts' Urine Strain absorbed with Hirschfeld Strain (dose, 7 billion per c.c. Undiluted Serum).

Strains used in test.	Titres of unabsorbed control.	Titres after absorption.
Aertrycke: Newport	5,500	0 (at 1 : 125)
Aertrycke: Mutton	5,600	0
Paratyphoid B	7,600	0
Hirschfeld strain	11,000	0
Witts' urine strain	11,000	0
Swine fever.	10,000	0
B. Voldagsen	8,500	0
B. Glässer	2,600	0

The reciprocal absorption gave quite similar results. We found that these paratyphoid C sera required very large doses to effect complete absorption; the Hirschfeld serum showed peculiar obstinacy in this respect. Thus, in absorptions of Hirschfeld serum with its own bacillus, a dose of 300,000 million per c.c. of serum produced little or no effect; a dose of 5 billion caused complete exhaustion.

When, on the other hand, we come to examine the behaviour of the paratyphoid C bacilli and their sera in relation to other members of the Salmonella group, a difference amongst them becomes apparent. As we shall show, our Witts' urine strain shows quite a marked relation with *B. paratyphosus B*, the Hirschfeld strain a less marked one and the Witts' blood strain almost none at all. Hence, in endeavouring to arrive at any conclusion as to the nature of *B. paratyphosus C*, it will be best to consider the last-named first, as presenting the "C" characters in their purest form.

A. *Serological Properties of the "Pure" Paratyphoid C (Witts' Blood Strain, when first Isolated).*

As regards agglutination and agglutinogenesis, the results shown in the following two tables indicate the remoteness of this strain from the paratyphoid B–Aertrycke group, and its clear relation to the *B. suipestifer* group. The figures given represent *percentage* agglutination, the titre of each serum for its own bacillus being taken as 100. They represent the averages of the figures obtained in many observations. The relatively poor agglutinability of Witt's blood strain makes the percentage figures for itself low, and those for other bacilli agglutinated by its serum high. Similarly the high agglutinability of the Witt's urine strain has precisely the opposite effect.

The first column in these tables on p. 167 shows that the "pure" paratyphoid C is not agglutinated at all by paratyphoid B and Aertrycke sera, and that its serum scarcely agglutinates the bacilli in question. On the other hand its relations with the suipestifer group, and notably with *B. Glässer*, are plain; but with Group II of the suipestifers (exemplified in the tables by *B. suipestifer* [Künzendorf] and *B. Voldagsen*) the relation is slighter, these bacilli being very feebly agglutinated by the Witts' blood strain serum.

The relations suggested by agglutination and agglutinogenesis are fully confirmed by mutual absorption tests, as we will now proceed to show. We have conducted nineteen quantitative absorption tests in which the Witts' blood strain

Percentage Agglutination of Paratyphoid C Strains by Salmonella Sera.

Sera.	Witts' blood strain.	Witts' urine strain.	Hirschfeld strain.
Aertrycke: Newport	0	30	39
Aertrycke: Mutton	0	33	46
Paratyphoid B	0	43	42
Paratyphoid C: Hirschfeld	56	92	100
Paratyphoid C: Witts' urine	45	100	100+
Paratyphoid C: Witts' blood	100	100+	100+
Swine fever (English)	37	52	57
Hog cholera (American)	28	30	47
B. suipestifer (Künzendorf)	31	45	50
B. Voldagsen	42	47	59
B. Glässer	82	100+	86

Percentage Agglutination of Various Salmonellas by Three Paratyphoid C Sera.

Strains tested.	Witts' blood strain serum.	Witts' urine strain serum.	Hirschfeld serum.
Aertrycke: Newport	1·5	56	5
Aertrycke: Mutton	1·7	58	7
Paratyphoid B	1·7	69	8
Paratyphoid C: Hirschfeld	100+	100+	100
Paratyphoid C: Witts' urine	100+	100	92
Paratyphoid C: Witts' blood	100	45	56
Swine fever (English)	100+	100+	100
Hog cholera (American)	100+	100+	100+
B. suipestifer (Künzendorf)	3	92	100
B. Voldagsen	3	99	86
B. Glässer	100+	29	67

NOTE.—The symbol 100+ indicates that, owing to differences in agglutinability, the titre was higher than that shown for the strain with which the serum had been prepared.

was used to absorb other sera, or in which its serum was absorbed with other Salmonellas: most of these have been reciprocal absorptions. The broad general results have been as follows: The paratyphoid C serum was exhausted of its specific agglutinin (more or less completely according to the dose of the absorbing organism) by other paratyphoid C's, by *B. Glässer* and, not quite so completely, by other members of suipestifer Group I (swine fever, Roy. Vet. Coll., and suipestifer 49 from America). The specific titre was not reduced at all in absorptions with suipestifers of Group II, with paratyphoid B, or with Aertrycke (Mutton and Newport). The specific agglutinin behaved in fact like a single entity: either it was quite unaffected or it was almost wholly absorbed. The serum used throughout was one made shortly after the isolation of the bacillus, and represented a "pure" paratyphoid C serum, but when we turn to the effects of the bacillus as an absorbing strain it must be remembered that, in the course of eighteen months, the organism gradually underwent mutation, finally approximating to the "urine strain." We could not, therefore, avoid the fallacy that we were operating with a less and less "pure"

paratyphoid C strain. With this proviso, it may be stated that the Witts' blood strain paratyphoid C was able to effect an almost complete exhaustion, when used in adequate dose, of other paratyphoid C sera, of Glässer serum, and of all suipestifer sera both Group I and Group II: the latter fact definitely affiliates the strain with Group I of the suipestifers. Contrary to expectation, paratyphoid B and Aertrycke sera were found to be definitely reduced in their specific titre by saturation with this strain of paratyphoid C, the reduction amounting to one half or even more. Within two months of its isolation, and before any mutation was observed, this strain reduced the titre of a paratyphoid B serum from 3600 to 2100. At a later date, after mutation had begun, it reduced the titre of a Mutton serum to 46 per cent. and of a Newport serum to 25 per cent. of the initial value. These facts are difficult of explanation, but suggest some paratyphoid B element, latent or actual, in the paratyphoid C strain under consideration.

We have sought for a compact diagrammatic method of expressing our absorption results and have adopted that seen below. The heights of the columns represent the actual and not the percentage titres of the serum, before absorption, for the different bacterial races. The shaded areas indicate the several titres left after the absorption; the unshaded areas represent the actual agglutinin absorbed. Gross inequalities in the titres of different sera are corrected by altering the scale to which they are drawn. Four examples of reciprocal absorptions have been selected for reproduction. When the columns of both members of a pair are left unshaded, or nearly so, complete homology is suggested between the strains concerned: if both were shaded total heterology would be suggested, but this is seldom the case. When one member of a pair shows shaded and the other unshaded columns, the fact indicates a one-sided absorption relationship such as subsists between Groups I and II of the suipestifers. Dosage of the absorbing strain is given in billions (*i. e.* million millions) per c.c. of undiluted serum. Doses below one billion have often proved insufficient to effect maximal absorption: doses of two billion and upwards have almost always proved sufficient to absorb all the agglutinin which can be absorbed.

These diagrams may be left to speak for themselves. It will suffice to summarise the evidence we have obtained as to the serological relationships of the "pure" paratyphoid C represented by the Witts' blood strain.

(1) In spite of the striking differences in cultural characters, its serological identity with *B. Glässer* (which, again, is serologically a member of Group I of the suipestifers) is almost quite complete. Maximal absorption of a Glässer serum with the paratyphoid C left only 3 per cent. of the Glässer agglutinin: the converse absorption left only 2 per cent. of the paratyphoid C agglutinin, while the Berlin strain of *B. Glässer* absorbed the paratyphoid C serum completely. Further evidence of serological identity is afforded by the facts that (*a*) the serum of each, allowing for differences in agglutinability, brings down the other to full titre; (*b*) in the most varied absorptions of other sera with other Salmonellas the titres for these two organisms invariably behave alike; (*c*) they exhibit the same one-sided relationship to members of the Group II of *B. suipestifer*.

(2) The relations with other members of Group I of the suipestifers are

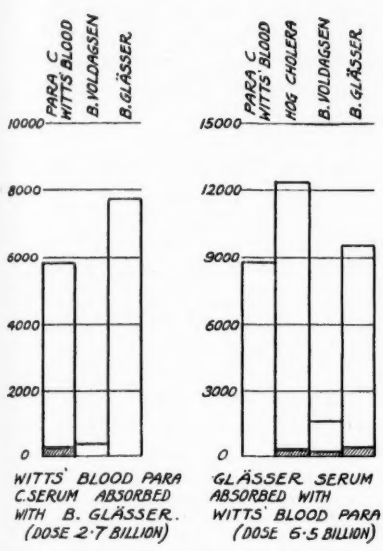

FIG. 1. RECIPROCAL ABSORPTIONS BETWEEN PARA C. (WITTS' BLOOD STRAIN) & B. GLÄSSER

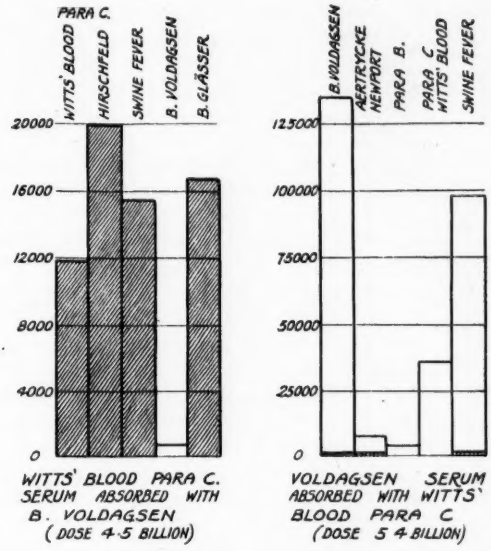

FIG. 2. RECIPROCAL ABSORPTIONS BETWEEN PARA C. (WITTS' BLOOD STRAIN) & B. VOLDAGSEN

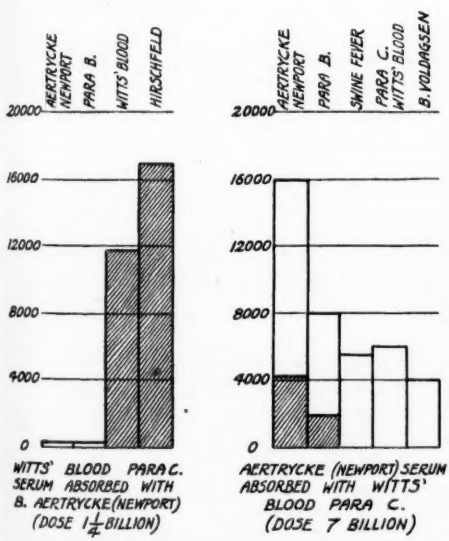

FIG. 3. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' BLOOD STRAIN) & B. AERTRYCKE (NEWPORT).

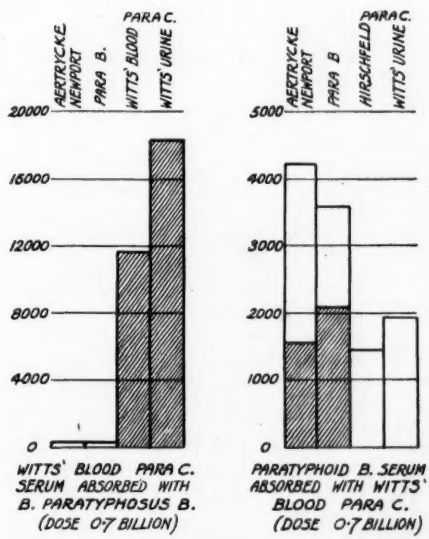

FIG. 4. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' BLOOD STRAIN) & PARATYPHOID B.

almost as close as with *B. Glässer*, though here again there are definite differences as regards the sugars fermented. As seen in the tables given on a previous page, the "pure" paratyphoid C serum brings down swine fever (hog cholera) strains to titre, and the sera of the latter have a very definite agglutinating power on paratyphoid C—really more than is indicated in the table because of the low agglutinability of the Witts' blood strain and the very high agglutinability of the suipestifer strains in question. Group I suipestifers will absorb nearly all the specific agglutinin from the paratyphoid C serum, only 3 to 4 per cent. being left. The reciprocal experiment was not carried out with this strain, but the Witts' urine stain absorbed swine fever serum well, leaving, in two experiments, only 8 per cent. and 4 per cent. of the specific agglutinin.

(3) With Group II of the suipestifers the relations are characteristic of Group I. Group II sera agglutinate the paratyphoid C fairly well; the paratyphoid C serum has but a feeble agglutinating power on members of Group II. Group II sera are exhausted of all their specific agglutinin on absorption with Witts' blood strain; but the pure paratyphoid C serum is wholly unaffected by large doses of Group II suipestifers (up to $4\frac{1}{2}$ billion).

(4) As regards relationship with paratyphoid B and the Aertrycke bacilli, they are very slight in the case of this strain. Mutual agglutination with their sera is absent or very weak. The specific titre of the serum of Witts blood strain is unaffected by very heavy saturation with paratyphoid B or *B. Aertrycke;* nevertheless, a potential relationship is suggested by the fact that Witts' blood strain even when first isolated did effect a distinct reduction of specific titre in a paratyphoid B serum, though this serum would not agglutinate it to any measurable degree. The potential relationship becomes an actual one when mutation occurs.

(5) No other relationships have been discovered. We have tested a Gärtner serum, a paratyphoid A serum and a typhoid serum on our paratyphoid C strains, and our paratyphoid C sera on these bacilli, with wholly negative result.

B. *Serological Properties of the Ordinary Strains of Paratyphoid C.*

The remaining strains of paratyphoid C which we have examined, representing what appears to be the more usual type, closely resemble the preceding in all respects, but differ in the intrusion of a paratyphoid B element to a variable extent, which leads to certain differences in serological behaviour. This shows itself in the following ways:

(*a*) These ordinary paratyphoid C strains are agglutinated by a paratyphoid B serum to something like a half of its full titre; they are similarly agglutinated by Aertrycke sera.

(*b*) Their sera agglutinate paratyphoid B and *B. Aertrycke* to a degree varying in its extent. Our paratyphoid C (Hirschfeld strain) serum acted only weakly on these bacilli, but the serum of our Witts' urine strain paratyphoid C agglutinated them to half or even two-thirds of its full titre.

(*c*) In absorption experiments there is seen evidence of a closer relation between these ordinary paratyphoid C's and the paratyphoid B-Aertrycke

group than was the case with the "pure" paratyphoid C strain. This is illustrated in the following graph, which represents reciprocal absorptions between *B. paratyphosus B* and the Witts' urine strain of *B. paratyphosus C.*

It is seen that this paratyphoid C strain removes about a quarter of the specific agglutinin from the paratyphoid B serum, and that the paratyphoid B strain removes half the specific agglutinin from the paratyphoid C serum. It is further of interest to note that whereas this is the case with the titre of the C serum for its homologous bacillus, the titre for the "pure" paratyphoid C (Witts' blood strain) is not affected at all by the absorption with paratyphoid B. This phenomenon was constantly observed in absorptions of Witts' urine strain serum by paratyphoid B or by Aertrycke strains. It may be effectively represented by a graph of a different kind showing the *percentage* reductions in its titres for different strains suffered by this paratyphoid C serum (Witts' urine strain) when absorbed with three different doses of paratyphoid B (fig. 6).

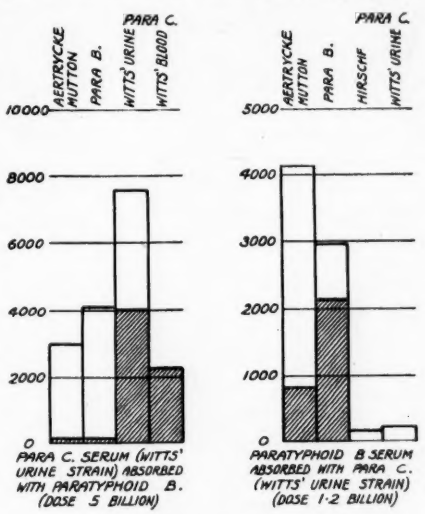

FIG. 5. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' URINE STRAIN) & PARATYPHOID B.

It is seen that the medium dose brings down the titre for the "compound" paratyphoid C's by some 50 per cent., no further reduction being effected by the very large dose; the titre for the "pure" paratyphoid C remains unaltered; that for paratyphoid B and *B. Aertrycke* is practically extinguished. One would be tempted to surmise that when such a compound paratyphoid C strain is agglutinated by its own serum, the effect is a composite one due to both C and B agglutinins, only the latter being removed by the absorption with paratyphoid B. We are, however, by no means disposed to argue from this single serum that all sera agglutinate in composite fashion by the resultant action of different components, for many facts are opposed to such a view.

It may be added that our "Witts' urine strain" of paratyphoid C appeared to contain a much larger paratyphoid B element than the Hirschfeld strain.

Only one further difference was detected between the pure paratyphoid C and the strains showing a paratyphoid B element. Whereas the serum of the former showed no reduction in its specific titre when absorbed with Group II suipestifers, the serum of our Witts' urine strain of paratyphoid C suffered a reduction of more than half, sometimes of more than three-quarters of its specific titre when so absorbed. There was never complete absorption. Group I suipestifers were able to absorb the agglutinin from this serum fairly completely, and the Hirschfeld serum was absolutely exhausted by *B. Glässer.*

THE MUTATION OF THE PURE INTO THE COMPOSITE *PARATYPHOID C.*

This phenomenon has been observed by most of those who have worked with this organism and we had the opportunity of following it in our own case. It has been explained that the strains isolated from the blood and from the urine differed in their relation to paratyphoid B. The urine strain was from the first agglutinated by a paratyphoid B serum to rather less than half titre, while paratyphoid B was agglutinated by the serum of this strain to $\frac{2}{3}$ of the titre of the latter for itself. No change was observed in the urine strain under 18 months' cultivation.

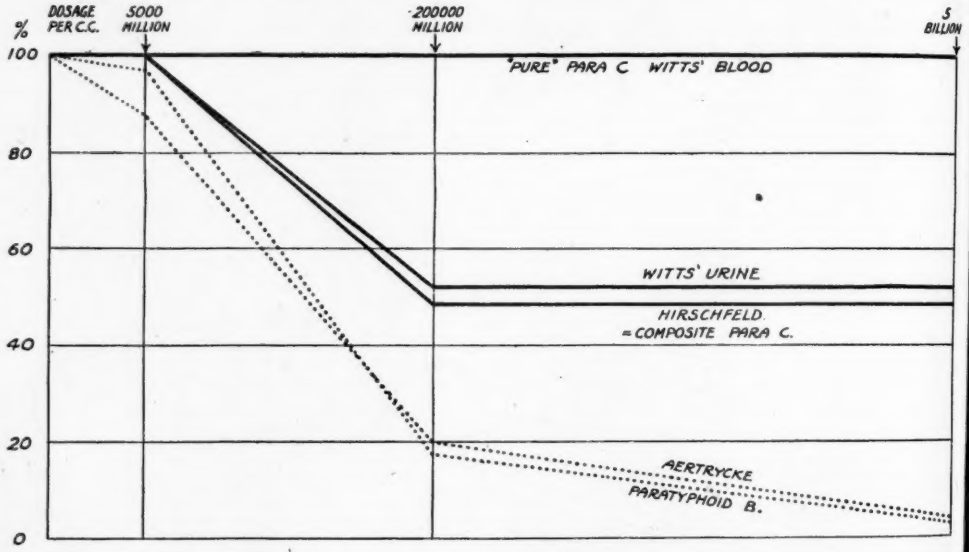

FIG. 6. PARATYPHOID C SERUM (WITTS' URINE) ABSORBED WITH 3 DIFFERENT DOSES OF B. PARATYPHOSUS B. SHEWING CURVES OF PERCENTAGE REDUCTION IN TITRE FOR DIFFERENT STRAINS.

It was otherwise with the blood strain. On first isolation it failed to agglutinate at all with a paratyphoid B serum (titre 1 : 5000) at all dilutions from 1 : 12½ upwards, and it was equally inagglutinable with Aertrycke sera. The strain was not inherently in-agglutinable, for it clumped freely with suitable sera. Our practice was to make a litre or so of formalinised broth culture and use this for all experiments; when it was used up a fresh stock was made, the culture having been kept up on egg-medium (only occasionally sub-cultured). Stocks of emulsion were made in July and December, 1919, and in July, 1920. In using the December stock it was noted that the strain showed some degree of agglutination with paratyphoid B and Aertrycke sera, and this was still more pronounced with the stock made in July, 1920. As we had some of all three stocks of emulsion available a comparison was made between them—all being put up simultaneously against sera of paratyphoid B and the Newport and Mutton types of *B. Aertrycke*. The readings were as follows :

Serum.	Emulsion 1. Made July, 1919, shortly after isolation.	Emulsion 2. Made Dec., 1919, after 5 mths'. culture.	Emulsion 3. Made July, 1920, after 12 mths'. culture.	Control with homologous bacillus.
Paratyphoid B.	Traces only	6,100 = 32 %	10,600 = 55 %	Para. B = 19,000
Aertrycke : Newport	„	2,210 = 13 %	6,100 = 38 %	Newport = 16,000
„ Mutton	„	512 = 12 %	1,235 = 28 %	Mutton = 4,400

It is seen that a progressive increase of agglutinability has occurred, most marked with the paratyphoid B serum. In the course of 12 months' subculture the blood-strain of our paratyphoid C has become serologically indistinguishable from the urine strain. Much is of course made of such facts as these by those who distrust serological classification, but it must be noted that this mutation of *B. paratyphosus C* involves no change in its essential specific agglutination; it is a side issue, involving only a co-agglutinin. Further, it is not a haphazard change, but an orderly and regular one seen by all who have had the opportunity of working with a "pure" paratyphoid C strain. The change is always in the same direction and does not pass a certain limit—that is to say, the C strain becomes agglutinable by a paratyphoid B serum to about 50 per cent. of the latter's full titre and not more; it does not become transformed into a paratyphoid B. Hirschfeld states that he has witnessed a reverse change, a strain of paratyphoid C, at first partially agglutinable with a paratyphoid serum, finally losing that property; this is the only instance of this which we have found recorded in the literature of the subject.

CONCLUSIONS.

(1) A new form of paratyphoid fever has been observed during the war by many investigators, chiefly in Eastern Europe and in Asia. The causal organism, *B. paratyphosus C*, is distinct from *B. paratyphosus A* and *B*, though presenting relationship to the latter.

(2) In this paper there is described a case of paratyphoid C occurring in England. The bacillus isolated is compared with other members of the Salmonella group in detail.

(3) In its cultural and fermentative characters *B. paratyphosus C* is shown to differ from *B. paratyphosus B* in its failure to ferment inosite and in its slower rate of alkali production. It differs from *B. suipestifer* in fermenting arabinose and dulcite.

(4) A short review is given of the serological properties of *B. suipestifer* and of the related forms *B. Voldagsen* and *B. Glässer*. We find the suipestifers to fall into two serological groups, sharply separable by certain sera. By absorption tests it is shown that a Group I suipestifer will exhaust the sera of both Groups I and II, but that a Group II suipestifer will exhaust only Group II sera, leaving the specific titre of Group I sera untouched.

(5) *B. paratyphosus C* is shown to belong serologically to the Group I suipestifers, in spite of the divergence in cultural characters.

(6) Different strains of *B. paratyphosus C* vary in their relation to *B. paratyphosus B*. In some the B element is absent, in others very obvious. An example of each variety was isolated from the case described, and it is shown that the strain which at first showed no relation to *B. paratyphosus B* gradually underwent a serological change, acquiring the property of being partially agglutinated by a paratyphoid B serum.

NOTE.

We do not make any claim to priority in affirming the relation of *B. paratyphosus C* to *B. suipestifer*. It was the knowledge that it could be agglutinated by Voldagsen and Glässer sera which induced us to compare it in detail with *B. suipestifer*. The division of the suipestifers into two serological groups is, we believe, novel; it enables us to associate *B. paratyphosus C* with one special group of suipestifers.

After the greater part of our work had been completed we read the paper of Dr. Carl Tenbroeck ([16]) on "Bacilli of the Hog-Cholera Group in Man." Tenbroeck had received two strains of *B. paratyphosus C* from Hirschfeld, and found on comparing them with *B. suipestifer* that there was strong serological correspondence, with complete mutual absorption. He adds a further piece of evidence: two rabbits immunised against Hirschfeld's paratyphoid C strains were found to be protected against a fatal dose of virulent hog-cholera bacilli.

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ON SOME CHARACTERS OF THE CLEAVAGE PRODUCTS OF CERTAIN BACTERIA, WITH SPECIAL REFERENCE TO THEIR TOXICITY AND ANTIGENIC PROPERTIES.

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Up to the present time the production of antibacterial sera, with perhaps the exception of that made from *B. dysenteriæ* (Shiga), has proved very difficult, and the sera, when made, have usually been found to give unsatisfactory results.

In making such sera the usual procedure is to inoculate horses with increasing doses of emulsions of either living or dead bacteria, but there is no generally recognised method used in their preparation or in testing their potency. For the latter purpose estimation of one or other of the so-called antibodies (such as the agglutinating, opsonic or bactericidal) has usually been employed; an example of this is the titration of the agglutinating power of antimeningococcus serum which is supposed to be a means of measuring its content of antitoxin.

Although the estimation of any of the antibodies probably gives some information concerning the degree of immunity attained in response to a series of inoculations, the very general belief that the antibodies are all formed together and that the estimation of one gives a comparative measurement of all, is not true. For the knowledge of the order and intensity in which antibodies are formed is still very incomplete, as is our knowledge of the nature and formation of the so-called endotoxins. This being the case, it appeared to be of fundamental importance to study these problems in any way possible.

In order to follow the *raison d'être* of the following experiments it will be necessary to consider what sequence of events follows on the inoculation of a bacterial vaccine, or a sublethal dose of living bacilli. Suppose, for instance, a dose of 500 million dead typhoid bacilli is injected under the skin of a man, it is known that shortly after the injection a considerable outpouring of lymph occurs at the site of inoculation, and that even in a previously uninoculated man this lymph has a considerable power of breaking down the bacteria and completely dissolving them. The solution of the dissolved bacteria is carried away in the lymph-stream, and if rapidly absorbed, as is the case when the inoculated man or animal takes active exercise or in any way stimulates the lymph-flow, causes a certain series of well-recognised symptoms, such as fever,

headache and general malaise. These symptoms rapidly subside, and, as has been frequently shown, are followed by an increased agglutinating, opsonic and lytic power of the blood-fluids, and under certain circumstances, far less well investigated, by protective power against the living organisms, and by the power to precipitate bacterial filtrates.

Taking these as the recognised facts, one essential for the production of the intoxication and general symptoms appears to be the lytic action of the blood-fluids which enables the broken-down bacteria to be carried in solution into the circulation, and it seems only reasonable to believe that unless the organisms of a vaccine are dissolved they cannot give rise to toxic symptoms, and that, only then, can antibodies be produced.

In a former research in conjunction with A. Fleming (1), it was shown that organisms which had been extracted with acetone were very readily attacked and dissolved by trypsin, while living suspensions of the same bacilli were little or not at all affected by this ferment, and it was further shown that these acetone-extracted organisms formed as good or even better vaccines as regards the production of antibodies than the unextracted bacilli killed by heat.

Another experiment showed that when typhoid bacilli were dissolved by means of normal human serum and the resulting mixture was centrifuged until a clear supernatant fluid was obtained, a perceptible precipitate was formed when this clear fluid was added to the serum of an animal that had received repeated doses of typhoid vaccines. This precipitin reaction was specific.

As a result of the conclusions drawn from these experiments, it was decided to ascertain the effect of inoculating acetone-extracted bacilli which had been acted on by trypsin, and also to examine such digested emulsions in respect of their toxicity and general antigenic properties.

THE EFFECT OF TRYPTIC DIGESTION ON THE TOXICITY OF BACTERIA.

Preparation of bacteria for digestion.—A series of agar cultures of a strain of *B. dysenteriæ* (Shiga) were grown in Roux bottles for 48 hours at 37° C. The growth was then washed off with just sufficient salt solution and the resultant emulsion divided into two parts. To both portions sufficient acetone was added, namely about 50 per cent., to bring about agglomeration and sedimentation of the bacteria. One portion was then placed in large tubes containing about 150 c.c. each and centrifuged until all the bacteria were caked into a mass at the bottom of the tube. The clear supernatant fluid was then decanted off, and the caked bacterial mass was spread over the sides of the tubes in an even, thin layer and afterwards placed in the incubator at 37° C. to dry. The dried scales were scraped off the sides of the tubes and preserved in a desiccator. The other portion of the emulsion was, after the addition of the acetone, allowed to sediment, and the supernatant fluid poured off as far as possible. Fresh pure acetone was then added, and when the organisms had again sedimented the supernatant acetone was removed. The concentrated sediment was then poured into a Soxhlet thimble, and, when the acetone had filtered away, the thimble containing the bacterial mass was placed in a Soxhlet apparatus and extracted with acetone for about forty hours. The contents of the thimble were then dried at 37° C. in the

incubator and these acetone-extracted organisms stored in powdered form in tubes. Preliminary experiments were then made comparing the toxicity of equal weights of the dried and acetone-extracted organisms. No difference could be detected, 0·075 mgrm. of each preparation killing rabbits of 1000 grm.

Method for preparing the digests.—Emulsions of these acetone-extracted bacilli were made in salt solution, each c.c. containing 10 mgrm. Two c.c. of such an emulsion were then placed in a dwarf test-tube and 100 c.mm. of a 1 per cent. sodium carbonate solution and 10 c.mm. of a sterile trypsin solution were added for each c.c., together with two or three drops of ether to prevent bacterial growth, and the tubes were sealed. Digestion was allowed to take place for about 4 to 18 hours at 37° C. As a result of the action of the trypsin the emulsion became almost completely clarified. In some experiments 10 per cent. of trypsin has been employed, but the results were almost identical.

The relation between the toxicity of the digests and the time of digestion.—This clear, or almost clear fluid was then tested for toxicity and it was found to kill rabbits of about 1000 grm. weight in doses representing 0·075 mgrm. of the acetone-extracted bacilli, when the digestion had been carried on for about eight hours at 37° C. The time that the trypsin is allowed to act appears to be an important factor. Numerous experiments were made and the following conclusions arrived at:

(1) Emulsions digested for short periods, such as 1 to 2 hours at 37° C. with 1 per cent. of trypsin, are not as toxic as an equal amount of the undigested emulsion.

(2) Emulsions digested for longer periods under similar conditions—for instance, 18 to 24 hours in the case of the strain employed in the experiments mentioned above—are also less toxic than the undigested emulsion.

(3) Different strains of *B. dysenteriæ* (Shiga) required different times of digestion to obtain the maximum toxicity, the optimum with the strain used in the above experiment being 8 to 10 hours at 37° C.

The relation between the toxin present in solutions of digested bacilli and that in suspensions of undigested bacilli.—The toxin obtained by digesting such emulsions gave rise to exactly the same symptoms as were produced by undigested bacilli, and the post-mortem appearances were also identical. To ascertain if the toxin, present in the digests, was the same as that which was derived from the intact bacilli, experiments were made to ascertain if an antitoxin, made by injecting horses with emulsions of untreated bacilli, completely neutralised the toxin contained in the digests.

The strain of *B. dysenteriæ* (Shiga) used in these experiments was somewhat deficient in toxic properties, and, in consequence, larger doses than usual had to be employed.

Experiment 1.

In the first experiment 5 rabbits, each about 1000 to 1250 grm., were used. Into No. 1, 0·5 mgrm. of an emulsion of acetone-extracted bacilli, which had been digested for about 4 hours at 50° C. with 10 per cent. trypsin, was injected intravenously. No. 2 was injected with 1·0 mgrm. treated in a similar manner. No. 3 received 5 mgrm., digested in the same manner, mixed with

0·5 c.c. of antitoxic serum three-quarters of an hour before the injection. No. 4 received 10 mgrm. treated in the same way, mixed with 1 c.c. of the same antitoxic serum. This mixture was also incubated for three-quarters of an hour at 37° C. before injection. No. 5 received 1 mgrm. of the acetone-extracted bacilli, which had *not* been digested.

The serum was obtained from Messrs. Burroughs Wellcome & Co., and was from a horse which had received injections of *B. dysenteriæ* (Shiga) only. The results of this experiment, which was carried out on October 4th, 1920, are shown in the following tables:

No. of rabbit.	Substances injected.	Result.
No. 1	0·5 mgrm. digested acetone-extracted bacilli	Partially paralysed on 8.10.20 and very nearly died, then slowly recovered.
No. 2	1·0 mgrm. digested	Died 8.10.20. Post mortem, typical lesions.
No. 3	5 mgrm. digested + 0·5 c.c. antitoxic serum	Died on 5.10.20. Post mortem, coccidiosis and tape-worm cysts found; rabbit very thin.
No. 4	10 mgrm. digested + 1 c.c. antitoxic serum	Remained quite well.
No. 5	1 mgrm. undigested acetone-extracted bacilli	Ill for 3 or 4 days, then recovered.

Experiment 2.

Six rabbits, each about 1000 to 1250 grm. in weight. Injections made on October 14th, 1920. Digestion of acetone-extracted bacilli carried on for 4 hours at 50° C. with 10 per cent. trypsin. All injections made intravenously. Results are shown in the following table:

No. of rabbit.	Substances injected.	Result.
No. 1	0·5 mgrm. digested bacilli	Died on 17.10.20. Post mortem, typical lesions.
No. 2	1·0 mgrm. digested bacilli	Died on 16.10.20. Post mortem, typical lesions.
No. 3	5 mgrm. digested bacilli, 0·5 c.c. antitoxic serum	Remained well.
No. 4	10 mgrm. digested bacilli, 1 c.c. antitoxic serum	Remained well.
No. 5	1 mgrm. acetone-extracted bacilli, undigested	Died 18.10.20. Post mortem, typical lesions.
No. 6	2 mgrm. acetone-extracted bacilli, undigested	Very ill for 2 or 3 days, but recovered.

The toxin and antitoxin mixtures were incubated for about one and a half hours at 37° C. before injection. This last experiment brings out two points: The first is that when digested for exactly the right time the digests of this strain are more toxic than the untreated bacilli. The second is that multiple

lethal doses of untreated bacilli sometimes fail to kill; this may be explained by the body-fluids, etc., failing to break down the bacteria into the toxic "phase."

As already mentioned, these experiments were carried out with *B. dysenteriæ* (Shiga), but numerous experiments were also made with a strain of *B. typhosus* with very similar results; thus dried and acetone-extracted bacilli killed mice when similar quantities of the organisms (namely 1 mgrm.) were injected intra-peritoneally. The same emulsion of acetone-extracted bacilli digested for about 18 hours at 37° C., or for somewhat longer periods at room temperature with 10 per cent. trypsin, invariably killed in doses of 1 mgrm., and frequently in smaller doses such as 0·5 mgrm. or even less. The signs of disease produced during life and the post-mortem appearances were similar; the latter consisted of an enlarged, deeply-coloured spleen, congested adrenals and an extensive necrosis of the intestine.

Experiments made with acetone-extracted meningococci also showed that emulsions, after being digested with trypsin, had a considerable toxicity; for instance, doses of 0·1 mgrm. or even less were found to be fatal when injected subdurally into guinea-pigs, producing a typical and constant series of symptoms and giving rise to constant *post-mortem* changes. The full details of these experiments and methods for testing the antitoxic value of meningococcic sera, etc., form, however, a special research, which is being undertaken in conjunction with Dr. Colebrook.

On the question of the toxic substance being formed during the process of digestion.—On consideration of all the results obtained it would appear probable that the actual toxic substance is produced during the digestion of the organisms, whether by the body-fluids or by trypsin. If, however, the digestion is carried on for too long a period, this substance is further broken down and the solution becomes less toxic. As the cleavage must continue during practically the whole period of digestion, only a proportion of the whole available toxin that could be theoretically produced from a given quantity of the dried bacterial substance can be present in solution at any given time.

Amongst the facts pointing to the toxin being produced *in vitro* by breaking down the bacterial proteids by the action of the tryptic ferment, the following may be mentioned:

(i) The toxic solutions, however concentrated, only give opalescence on the addition of half their volume of 33 per cent. trichloracetic acid.

(ii) After the injection of digests of *B. dysenteriæ* or *B. typhosus*, even in sublethal doses, diarrhœa is produced in a very short time, such as 30 minutes, while when undigested bacteria are employed, in similar doses, no diarrhœa is noticed until after the lapse of a much longer period.

(iii) Different varieties of bacteria, and perhaps different strains of the same organisms, give the maximum toxicity after varying times of digestion.

(iv) From time to time it has been noticed when giving injections of dead bacilli that a certain dose will always kill the animal, but if a larger dose, perhaps two or three times as large as is necessary to kill the animal, is given, the animal survives. Such a fact has been recorded by Gordon [3] in the case of the meningococcus, and, in one of the experiments given in detail above, 2 mgrm. of dried *B. dysenteriæ* (Shiga) failed to kill, whereas, in this experi-

ment and several others, 1 mgrm. proved to be the lethal dose for rabbits of the size used in the experiments.

The most satisfactory explanation of these facts is that when the dose surpasses a certain amount the animal is unable to break down the bacteria into the toxic "phase," and, in consequence, is able to survive a dose capable of killing several animals.

If the digests were poured into ten times their volume of alcohol a heavy precipitate was formed, which was easily collected by centrifuging. This precipitate which had remained in contact with the alcohol for from seven to ten days so as to coagulate all the free proteid present, and which had been washed free of some of the amino-acids and other substances by dialysis under pressure through a collodium membrane, was extracted with faintly alkaline salt solution, and this extract filtered was found to be still strongly toxic—in fact the alcoholic precipitate was much more toxic than an equal weight of the original dried bacilli. This shows that the toxin was not a proteid body capable of being coagulated by alcohol.

THE ANTIBODY RESPONSE TO INJECTIONS OF DIGESTED BACTERIA.

Experiments were carried out to amplify, and confirm, the information derived from a single experiment, the results of which were given in a paper written in conjunction with A. Fleming (1), in which the digested organisms were used as the antigen for inoculating animals, and to ascertain more exactly what response these animals made to such injections.

Experiment 1 : Injection of Digested Bacilli.

In the first experiment, the details of which are given below, *B. typhosus* was the organism employed.

Six rabbits of about equal weights were taken; No. 1 and No. 2 were inoculated throughout the experiment with acetone-extracted bacilli; No. 3 and No. 4 with acetone-extracted bacilli which had been digested overnight with 10 per cent. trypsin at 37° C.; No. 5 and No. 6 with dried typhoid bacilli). All the inoculations were given intravenously.

On 31.12.20 the amount injected was 0·1 mgrm.

On 4.1.21, 0·3 mgrm. was inoculated.

On 13.1.21 a sample of blood was taken from each animal, and the agglutinating and bactericidal powers were estimated, with the following results:

Agglutinating Power of Sera.

No. of rabbit.	Dilutions of serum. 1 in:									
	25	50	100	200	400	800	1600	3200	6400	12,800
Rabbit No. 1	+	+	+	+	+	+	+	—	—	—
„ No. 2	+	+	+	+	+	+	+	S.	—	—
„ No. 3	—	—	—	—	—	—	—	—	—	—
„ No. 4	—	—	—	—	—	—	—	—	—	—
„ No. 5	+	+	+	+	+	+	+	+	+	S.—
Rabbit No. 6	+	+	+	+	+	+	+	S.+	—	—

In estimating the agglutinating power of the serum Dreyer's method was employed, and the same emulsion, a formolised broth culture suitably diluted, was used throughout the whole series of experiments.

Bactericidal Power (Wright's Method).

Dilutions of culture.	Control normal rabbit.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.	Rabbit No. 5.	Rabbit No. 6.
1 in 1,000,000 . .	Growth	Growth	Sterile	Sterile	Sterile	Sterile	Growth.
1 in 100,000 . .	„	„	Growth	„	„	Growth	„
1 in 10,000 . .	„	„	„	„	„	„	„
1 in 1000 . .	„	„	„	„	Growth	„	„
1 in 100 . .	„	„	„	Growth	„	„	„
1 in 10 . .	„	„	„	„	„	„	„

Ten c.mm. of diluted culture mixed in each case with 10 c.mm. of serum (Wright's method). Culture contained 480 million living bacilli per c.c.

A further test was made on 17 . 1 . 21 of the agglutinating, bactericidal and opsonic powers of the serum of each animal.

Agglutinating Power.

No of rabbit.	Dilutions of serum. 1 in: 25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000
No. 1 . . .	+	+	+	+	+	+	S.	—	—	—	—	—
„ 2 . . .	+	+	+	+	+	+	+	S.+	—	—	—	—
„ 3 . . .	S.—	—	—	—	—	—	—	—	—	—	—	—
„ 4 . . .	—	—	—	—	—	—	—	—	—	—	—	—
„ 5 . . .	+	+	+	+	+	+	+	+	S.+	—	—	—
„ 6 . . .	+	+	+	+	+	+	+	S.+	—	—	—	—

Bactericidal Power (Wright's Method).

Dilutions of culture.	Control normal rabbit.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.	Rabbit No. 5.	Rabbit No. 6.
1 in 1,000,000 . .	Growth	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile.
1 in 100,000 . .	„	Growth	„	„	„	„	Growth.
1 in 10,000 . .	„	„	„	„	„	„	„
1 in 1,000 . .	„	„	Growth	„	„	Growth	„
1 in 100 . .	„	„	„	„	„	„	„
1 in 10 . .	„	„	„	Growth	Growth	„	„

The culture contained 520 million living bacilli per c.c.

Opsonic Power.

The sera of rabbits Nos. 1, 2, 5 and 6 were all found to have very greatly increased opsonic power, while those of rabbits Nos. 3 and 4 remained almost within normal limits.

These results show that the inoculation of the acetone-extracted bacilli and dried bacilli produced practically identical results, namely an increase of the agglutinating, opsonic and bactericidal powers, while the inoculation of the digested acetone-extracted bacilli produced a very marked rise of the bactericidal power greater than did either the acetone-extracted or the dried bacilli, but *no* increase of the opsonic or agglutinating powers.

A further series of intravenous inoculation was administered to the same animals.

On 19.1.21, 0·5 mgrm. was inoculated.

On 24.1.21, 1·0 mgrm. was inoculated.

On 25.1.21, rabbit No. 1 died of gas gangrene.

On 28.1.21, 2·0 mgrm. were inoculated into the surviving five rabbits.

A sample of blood taken before this inoculation showed the agglutinating power of No. 2, No. 3 and No. 4 to be as follows:

Agglutinating Power.

	Dilutions of sera. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,600	50,000	100,000
No. 2	+	+	+	+	+	+	+	+	+	+	S.—	—	—
„ 3	—	—	—	—	—	—	—	—	—	—	—	—	—
„ 4	—	—	—	—	—	—	—	—	—	—	—	—	—

The inoculations were continued.

On 31.1.21, 4·0 mgrm. were inoculated.

On 4.2.21, 8·0 mgrm. of the bacilli were injected.

A sample of blood was taken from rabbits No. 2, No. 3 and No. 4 before the inoculation, and the agglutinating and precipitating powers were tested.

Agglutinating Power.

	Dilutions of sera. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2	+	+	+	+	+	+	+	+	+	+	S.+	—	—
„ 3	+	S.—	—	—	+	+	—	—	—	—	—	—	—
„ 4	Tr.	—	—	—	—	—	—	—	—	—	—	—	—

Precipitating Power.

In these experiments the antigen employed was the filtrate of a digested emulsion of acetone-extracted bacilli containing 5 mgrm. per c.c., the digestion being carried out at 37° C. with 1 per cent. trypsin overnight. One hundred c.mm. of serum was added in each case to 1·0 c.c. of the diluted antigen. The amount of precipitate formed is roughly indicated by the number of crosses.

	Dilutions of antigen. 1 in:					
No. of rabbit.	Undil.	2	4	8	16	32
No. 2	+++	++	+	Trace+	Trace—	Haze
No. 3	++	+++	+++	++	+	+
No. 4	++	+++	+++	++	+	+

These results indicate that the inoculation of a series of doses from 0·1 up to 4·0 mgrm. of the digested acetone-extracted bacilli produced practically no increase of the agglutinating power above the normal, while the precipitating power was increased considerably more than in the case of the rabbit which received the undigested acetone-extracted bacilli.

The inoculations were continued.

On 7.2.21, rabbits No. 2, No. 3, No. 4, No. 5 and No. 6 received doses of 16 mgrm.

On 14.2.21 a sample of blood was taken from each animal, and the agglutinating power was estimated with the following results:

Agglutinating Power.

	Dilutions of serum. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2.	+	+	+	+	+	+	+	+	+	+	+	+	Tr.+
„ 3.	+	+	+	+	+	Tr.+	—	—	—	—	—	—	—
„ 4.	+	+	Tr.	—	—	—	—	—	—	—	—	—	—
„ 5.	+	+	+	+	+	+	+	+	+	+	+	+	S.+
„ 6.	+	+	+	+	+	+	+	+	+	+	+	+	S.+

Bactericidal Power (Wright's Method).

All the sera were found to be either normal or below normal in respect of the bactericidal power. It was noted that No. 5 and No. 6 had even less power than the normal animal.

Precipitating Power.

The same technique and antigen was used as in the former experiments.

	Dilutions of antigen. 1 in:					
No. of rabbit.	2	4	8	16	32	64
No. 2.	++	+	+	±	—	—
„ 3.	+++	+++	++	++	+	±
„ 4.	+++	+++	+++	++	+	Tr.
„ 5.	++	++	++	+	Tr.	—
„ 6.	++	++	++	+	+	—

The inoculations were continued.

On 18.2.21, 20 mgrm. were inoculated.

On 25.2.21, 20 mgrm. were inoculated.

On 25.2.21, No. 6 died.

On 4.3.21, samples of blood were taken from the surviving four rabbits, and the sera was tested for the agglutinating, opsonic and precipitating powers.

Agglutinating Power.

	Dilutions of serum. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2.	+	+	+	+	+	+	+	+	+	+	+	+	—
„ 3.	+	+	+	+	+	+	—	—	—	—	—	—	—
„ 4.	+	+	+	+	+	+	S.	—	—	—	—	—	—
„ 5.	+	+	+	+	+	+	+	+	+	+	+	+	S.+

Opsonic Power.

By the dilution method No. 2 and No. 5 were found to be enormously increased, while No. 3 and No. 4 were almost within normal limits.

Precipitating Power.

The same antigen was employed, but to have the same quantitative measurement the technique consisted in adding 0·5 c.c. of the serum to 1 c.c. of the antigen in a tube drawn out to a contracted end. The precipitate was allowed to settle for 24 hours after being in a bath at 50° C. for 4 hours, and the tubes were centrifuged until the precipitate was tightly packed. The tube was marked at the upper border of the precipitate, then cleaned out thoroughly, dried and measured amounts of mercury run in until it occupied the same bulk as the precipitate.

The results were as follows:

Rabbit No. 2 gave 5 c.mm. of precipitate; rabbit No. 3, 10 c.mm.; rabbit No. 4, 8 c.mm., and rabbit No. 5 only a trace less than 2 c.mm.

These latter results show that a slight amount of agglutinating power had been produced in the serum of the animals inoculated with the digested acetone-extracted organisms. This was most probably due to the fact that a small percentage of the organisms escape digestion, so that when large doses, such as 20 mgrm., of the bacilli are injected, a sufficient number remain undigested to produce a comparatively slight increase in the agglutinating power.

The opsonic power of No. 3 and No. 4 remained almost normal, while the precipitating power of these two animals was increased considerably more than No. 2 or No. 5, which had received undigested organisms.

Summary of the Results of this Experiment.

It will be noted that in response to the earlier and smaller injections no increase of agglutinating power was developed in the sera of the animals injected with the digested organisms, but, when the larger doses of digested organisms were employed, some slight increase of the agglutinating power was produced. Almost exactly similar results were observed as regards the opsonic power, but the bactericidal power and precipitating power were largely increased.

Experiment 2.—Injection of Filtered Digested Bacilli.

Further experiments in which filtrates or thoroughly centrifuged digested emulsions of acetone-extracted bacilli were used as the antigen for intravenous injections showed that no increase of agglutinating or opsonic power was brought about, even after repeated injections, while the bactericidal and precipitating powers were much raised. The details of this experiment, in which *B. typhosus* was again employed, were as follows:

Three rabbits were taken, two of which—No. 1 and No. 3—had some months before received one or more doses of filtrates of digests of acetone-extracted bacilli. Rabbit No. 2 had never received any previous injections.

On 17.3.21, Nos. 1 and 2 received intravenously 0·1 c.c. of an emulsion of acetone-extracted typhoid bacilli, 5 mgrm. to 1 c.c., which had been digested overnight at 37° C. with 1 per cent. trypsin and then filtered through rough paper pulp.

On 18.3.21, No. 3 received 0·5 c.c. of the same filtrate.

On 21.3.21, No. 1 and No. 2 received 0·25 c.c., and No. 3, 0·5 c.c. intravenously of the same filtrate.

On 30.3.21, No. 1, No. 2 and No. 3 each received 0·5 c.c. intravenously.

On 2.4.21, No. 1, No. 4 and No. 3 each received 1 c.c. intravenously.

On 6.4.21, No. 1, No. 2 and No. 3 were given 2 c.c. intravenously.

On 9.4.21, No. 1, No. 2 and No. 3 were each inoculated intravenously with 1 c.c. of the filtrate, which had been concentrated to about one-fourth of its volume by filtration through a collodium membrane.

On 12.4.21, a sample bleeding was made.

None of the sera agglutinated a sensitive emulsion of *B. typhosus* in 1 in 25 dilution.

Precipitating power was well developed. A filtrate of a digested emulsion of acetone-extracted *B. typhosus* was used as the antigen.

	Dilutions of antigen. 1 in.:				
No. of rabbit.	2	4	8	16	32
No. 1.	++	++	+	Trace	—
„ 2.	++	+	Trace	—	—
„ 3.	++	++	+	+	Trace

Bactericidal Power.

Dilutions of culture.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Control normal.
1 in 1,000,000	Sterile	Sterile	Sterile	Sterile
1 in 100,000	„	„	„	„
1 in 10,000	„	„	„	Growth
1 in 1,000	Growth	„	Growth	„
1 in 100	„	Growth	„	„
1 in 10	„	„	„	„

The inoculations were continued.

On 13.4.21, No. 1, No. 2 and No. 3 each were inoculated with 1·75 c.c. of the concentrated filtrate.

On 16.4.21, No. 1, No. 2 and No. 3 were inoculated intravenously with 1 c.c. of a filtrate which had been precipitated with alcohol and re-dissolved in saline.

On 22.4.21 a further sample bleeding.

Agglutinating power was absent from all the sera in 1 in 25 dilution.

Precipitating Power.

The precipitating power is shown below. The antigen employed was a solution of the digest which had been precipitated with alcohol and re-dissolved in saline.

	Dilutions of antigen. 1 in:					
No. of rabbit.	10	20	40	80	160	320
No. 1.	+++	+++	++	++	Trace +	Trace —
„ 2.	+++	++	+	Trace	—	—
„ 3.	+++	++	++	++	Trace	—

On 23.4.21, No. 1, No. 2 and No. 3 each were inoculated intravenously with 1 c.c. of the same solution as was given on 16.4.21.

On 29.4.21 a further sample bleeding.

Agglutinating Power.

Serum of No. 1 agglutinated to standard + in 1 to $2\frac{1}{2}$ dilution, and gave a trace in the 1 in 5, but no signs of agglutination in 1 in 10 or higher dilutions.

No. 2 gave trace in 1 in $2\frac{1}{2}$, but nothing in any higher dilution.

No. 3 gave standard + in 1 in $2\frac{1}{2}$, nothing in the higher dilutions.

These agglutinating powers, it may be noted, fall well within the normal limits.

Precipitating Power.

Same antigen as in the last experiment.

	Dilutions of antigen. 1 in:					
No. of rabbit.	5	10	20	40	80	160
No. 1.	+++	+++	++	+	±	Tr.—
„ 2.	+++	++	+	±	—	—
„ 3.	+++	+++	++	+	±	Tr.—

On 4.5.21 a further sample bleeding was taken.

Agglutinating Power.

	Dilutions of antigen. 1 in:					
No. of rabbit.	$2\frac{1}{2}$	5	10	20	40	80
No. 1.	S.	—	—	—	—	—
„ 2.	S.+	—	—	—	—	—
„ 3.	S.+	—	—	—	—	—

Precipitating Power.

The same antigen was employed.

	Dilutions of antigen. 1 in:					
No. of rabbit.	10	20	40	80	160	320
No. 1.	+++	+++	++	+	Trace	—
„ 2.	+++	+++	++	+	„	—
„ 3.	+++	+++	++	+	„	—

To obtain a more accurate gauge of the precipitating power of these sera 0·5 c.c. of each serum was mixed with 1 c.c. of a digest, and after standing overnight the tubes were centrifuged and the volume of the precipitate measured by means of mercury.

It was found that No. 1 gave 11 c.mm. of precipitate, No. 2 gave 8 c.mm. and No. 3 gave 11 c.mm.

This experiment therefore shows that no agglutinins are formed when the digests are freed from undigested bacteria.

PRECIPITABLE SUBSTANCE PRESENT IN DIGESTS OF ACETONE-EXTRACTED BACTERIA.

While carrying out some of the above experiments it was observed that, on mixing the digests of organisms with the corresponding antitoxic serum a considerable precipitate was formed, and on making further tests this

precipitate was found to be specific, and the phenomenon was, in fact, a typical specific precipitin reaction.

Further experiments showed that digests of not only *B. dysenteriæ*, but also of *B. typhosus*, meningococcus, tubercle bacilli and other organisms, filtered through paper pulp or a Berkfeld filter until perfectly clear, gave heavy precipitates on the addition of sera obtained from animals which had received repeated injections of the bacteria in question.

In addition to these results a large number of experiments were made in which human serum, after being digested until only a small amount of precipitate was formed on the addition of 33 per cent. trichloracetic acid, was mixed in various dilutions with a constant volume of the serum of a rabbit which had received several injections of unaltered human serum. A copious precipitate was formed with certain dilutions, but it was found that, when a comparison was made of digested and undigested serum, the undigested sample could always be diluted to a higher degree than the digested serum before the end-point of precipitation was reached. This appears unavoidable, as whatever substances produce the precipitate on being mixed with the immune serum, a proportion is destroyed owing to the length of time the digestion must be carried on, since, as in the case of the endotoxins, prolonged digestion leads to the destruction of the precipitable substance. When digests of bacteria are precipitated with alcohol for 7 to 10 days so as to coagulate any unaltered albumens, a filtered extract of this precipitate gives rise to a precipitate on the addition of the corresponding immune serum. In the case of digests of bacteria the precipitate formed was often abundant, and the extract of the alcoholic precipitate could be greatly diluted before an end-point was reached, but when a similar experiment was made using digested blood-serum the precipitate obtained was very much smaller in bulk, and was much more slowly formed than when unaltered serum was used in the test. Control experiments made by digesting serum obtained from other mammals showed this precipitate to be specific. The precipitates, whether they were obtained from a digest of bacteria mixed with the corresponding immune serum or with serum mixed with its appropriate antiserum, were constantly found to be insoluble in solutions of trypsin, even when washed free from all unaltered serum and suspended in alkaline salt solution. The significance of this fact will be discussed in a later section.

The production of the precipitating power in animals.—To ascertain if a specific precipitating power to albuminous fluids, such as blood-serum, was produced in the serum of rabbits, as the result of intravenous injection of serum, which had been digested until practically all the proteid constituents had been broken down by trypsin, rabbits were inoculated intravenously with human blood-serum, which had been digested with a large excess of trypsin until only a very slight precipitate was formed on the addition of 33 per cent. trichloracetic acid. After a rabbit had received two intravenous injections, representing 3 c.c. of serum in all, the animal produced a serum which had a power of producing the specific precipitate when mixed in the proportion of 100 c.mm. of this rabbit serum to 1 c.c. of diluted untreated human serum, up to a dilution of 1 in 1280.

After human blood-serum had been digested and the resulting solution had

been precipitated with 10 volumes of alcohol the precipitate was extracted with saline and passed through an ordinary filter-paper. This clear solution, as has been shown above, when mixed in suitable amounts with anti-human rabbit's serum gave a distinct although slight precipitate. When, however, it was employed as the antigen and injected intravenously into a rabbit no development of precipitating power could be detected. The details of this experiment are given below:

The rabbit received intravenously first the re-dissolved precipitate from 1 c.c. of blood-serum, the next day that from 3 c.c., and 3 days later the remainder of the precipitate representing 6 c.c.—in all the precipitate from 10 c.c. of blood-serum. Sample bleedings were taken 6 and 10 days after the last injection, but no precipitating power could be demonstrated.

From the above experiments it appears probable that the precipitating power of the serum which is produced in response to injections of bacteria or serum, is brought about by the reaction of the body, *not* to the unaltered proteids of the bacteria or serum, but to some degradation product of these proteids which is produced by the action of the proteolytic ferments present in the blood-fluids when untreated bacilli are injected.

SIMILAR WORK CARRIED OUT BY OTHERS.

Experiments of somewhat similar character, resulting in various conclusions, have been carried out by other workers. For instance, R. Pfeiffer (3) maintained that the lytic action of the body-fluids caused the toxicity of several species of bacilli; Vaughan (4) held that the endotoxins were degradation products of the bacterial protoplasm; and many other workers have investigated the toxic properties of certain bacteria, usually *B. typhosus*, by breaking down the bacilli either by mechanical means (Macfaydean) (5), or by the lytic action of the serum (Besredka) (6). More recently Jobling, using ferments obtained from leucocytes (leuco-proteases), came to the conclusion in his early work (1912) (7) that the cleavage products of pneumococci were capable, when injected into animals, of giving rise to a specific opsonic increase, while in a later work (1913) (8), in which *B. typhosus* was the organism employed, he concluded that although the toxic substance which is produced when the bacilli are broken down with leucoprotease is the same as that which results from the breaking down of the bacilli by the lytic action of the blood-fluids, etc., *in vivo*, yet it is not the true endotoxin, since the same symptoms and lesions can be produced when other bacilli are employed. Quite recently (1920) Sanarelli (9), working with cultures of *V. choleræ* partially digested with trypsin, came to the conclusion that the specific endotoxin, which he believes is present in the intact vibrio, is set free during the digestion, and produces, when injected intravenously into guinea-pigs in sufficient quantity, a condition very similar to the disease seen in man. Further, he believes that these digested cultures form as complete an antigen as the undigested bacilli, and are capable of producing all the recognisable antibodies.

When these results and conclusions are contrasted with those obtained in the present research it is seen that in the case of *B. dysenteriæ* (Shiga) the toxin present in the digests is the specific endotoxin, and that this is also the

case with meningococcus; here the specificity is so marked that some difference could be detected between the various types. The details of these experiments, which were carried out in conjunction with L. Colebrook, will be given in a later publication. As regards the digests forming as complete an antigen as the undigested organisms, this research shows conclusively that no increase of the agglutinating or opsonic powers is produced in response to injections of digested bacteria, if care is taken to exclude bacteria on which the ferment has not acted.

DISCUSSION.

On consideration of the various results it may be stated with a considerable degree of certainty that the agglutinating and precipitating powers of a serum, instead of being connected very closely with one another, are in reality widely separated, being in all probability produced in the body in response to very different stages of the cleavage of the bacteria proteids.

It would also appear probable that, when intact bacteria are injected into an animal, the agglutinating and opsonic powers are produced in response to a very early stage of the dissolution of the bacteria by the tissue fluids. At a later stage the lytic powers are developed; a still more advanced stage of the same process produces the specific "endotoxin" to which the body reacts by the production of the antitoxin; lastly, and very nearly connected with this toxic substance, is that in response to which the precipitating power is developed. By the last reaction the cleavage products of the foreign proteid are returned to an insoluble phase which is incapable of being acted on by such ferments as trypsin, and most probably by those ferments present in the body-fluids, which appear to act in a similar manner. The removal of this precipitate from the circulation is possibly carried out by the leucocytes, which then settle in certain of the internal organs. Its final disposal is carried out in some manner at present unknown, but not improbably from the mucous surfaces by a process similar to that whereby the excess of lymphocytes is disposed of.

It appears advisable to point out some of the ways in which bacteria which have been broken down by trypsin may prove of practical value. Bacteria broken down to the toxic stage appear to be in the best form (i) for testing the antitoxic value of any antibacterial serum, since their use entirely overcomes the difficulty of employing multiple lethal doses, which, when the undigested bateria are employed, sometimes fail to kill. (ii) The toxic products may be considerably increased in potency by such methods as precipitation with alcohol, removal of the amino-acids, etc.—the details of which will be given later. They are therefore in a much better form for inoculating into animals for the production of antitoxin, and it is to be hoped that by their employment more efficient antitoxin may be obtained. (iii) In response to injections of the digests certain of the antibodies, which further experiments may increase in number, are not developed. It is therefore possible that the mechanism whereby the remaining antibodies are produced in the body will not be so easily exhausted, and, in any case, the bacterial proteid, having been broken down to the toxic stage outside the body, the disappearance of the lytic power as a result of numerous injections will not be of the same importance.

If it is true that—in accordance with the theory propounded above—the toxicity of some organisms is due to their proteid constituents being broken down by the proteolytic ferments of the body-fluids, it is obvious that, when these are rendered less active, as gauged by the diminished bacteriolytic power, the antitoxic power cannot be increased above a certain range. However many injections of bacilli may be given, very little or none of their proteid constituents will be presented to the body-cells and fluids in the "phase" suitable to produce an "antitoxic response"; this would also apply to the production of precipitating sera. If, however, before the injections are given, the proteids of the bacilli are broken down outside the body to the suitable "phase," there should be no limit to the range of the antitoxic and precipitating response, and an improvement in the production of anti-endotoxic sera may follow the use of these digests in immunisation.

Digests of bacteria form excellent antigens for employment in tests for complement-deviation, and by their use it may be possible to ascertain when certain chronic diseases are in an active condition, since when a bacterial infection is progressive some of the bacteria must come into contact with the body-fluids and cells. In consequence, degradation products of the bacterial proteids will be produced, and the body will develop an increase of the anti-bodies in response to these, which should be detected by complement-deviation tests in which a suitable digest is used for the antigen. If, however, the disease becomes arrested completely no bacteria will be acted on in this way, and therefore the antibody content of the blood against such cleavage products should fall to within normal limits.

Digests of bacteria may also prove of use as vaccines, especially therapeutically. The type of patient which may be benefited by their use is that which has become "tolerant" to the ordinary vaccine made by killing the bacteria by heat. Such subjects appear to be unable to break down the vaccine into the stage necessary for the development of antibodies, and might be greatly benefited by inoculation of suitably broken-down bacteria, especially where there is no evidence of autoinoculation.

I should like to thank Dr. W. Gye for kindly criticism, and Dr. L. Colebrook for carrying out several of the opsonic estimations.

SOME CONCLUSIONS.

Emulsions of acetone-extracted bacteria which have been digested with trypsin for a suitable period have a toxicity which is the same as, or at times greater than, that of emulsions of untreated bacteria of equal density.

The toxin present in the tryptic digests is the specific bacterial endotoxin which is usually produced in the body by the lytic action of the body-fluids.

Antitoxin prepared by the injection of untreated bacteria completely neutralises the toxic properties of the tryptic digests.

When bacterial digests are injected into animals the bactericidal and precipitating powers are greatly increased. Antitoxin is also formed, but there is no increase of either the agglutinating or opsonic powers.

This shows that the agglutinating and precipitating properties of the serum, instead of being very closely related or even identical, are quite distinct from one another.

It is also suggested that the antigens giving rise to the agglutinating and opsonic powers are produced during the very early stages of the solution of the bacteria by the body-fluids, while the antigens, in response to which the body produces the bactericidal, antitoxic and precipitating powers, are formed during a comparatively late period of the lytic process.

Repeated injections of untreated bacteria cause diminution of the lytic power of the blood-fluids even when comparatively small doses are employed. This would account for the difficulty in producing potent anti-endotoxic sera, as the loss of the lytic power would prevent the injected bacteria being digested *in vivo* as far as the toxic phase, and, in consequence, no antitoxic response would be evoked.

Digestion of the bacteria *in vitro* presents the endotoxin preformed to the body, and so may lead to the production of much more potent antitoxic sera.

Digests of bacteria should prove to be more suitable for testing the potency of anti-endotoxic sera than emulsions of untreated bacteria, and since such digests contain large amounts of the specific precipitable substance they should be employed in estimating the precipitating properties of sera.

Other tests in which such digests may be employed are:

(i) As antigens in complement-fixation tests; here it is suggested that the activity of a disease might be recognised by the detection in the blood of antibodies to particular products of bacterial cleavage by using as the antigen the corresponding phase of a tryptic digest.

(ii) As vaccines for therapeutic use, especially in those subjects which have become "tolerant" to the ordinary vaccine—a condition in which the body appears to have lost all power of breaking down the bacteria of the vaccine.

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THE OCCURRENCE OF HÆMOLYTIC SUBSTANCES IN NORMAL URINE.

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In a paper recently published [1] it was pointed out that certain specimens of normal urine contained hæmolytic substances. The number of normal cases examined, however, was small, and the cases were not unselected; the results, therefore, could not be taken as representative of the frequency with which hæmolytic substances occurred in normal urine. An opportunity of examining a large number of specimens of the urine of normal persons has since occurred; and, though the results may be expressed very briefly, it is thought that their importance warrants their publication as a supplementary note to the paper referred to above.

Two hundred specimens of normal urine were available, each obtained from a healthy individual; in all cases the urine was fresh, and had not stood long after being passed. The method of estimating the hæmolytic activity was the same as that adopted in the paper mentioned above, to which the reader is referred for all details. The titre of a urine was taken as the smallest amount which could completely hæmolyse 1 c.c. of the standard blood suspension, when the mixture of blood and urine was incubated at 37° C. for four hours. Readings of results were taken after this period, and also at a period 2½ hours after incubation was begun.

The following were the results obtained:

(1) Of the 200 urines examined 19 per cent. produced hæmolysis of the suspension in 2½ hours, while 64 per cent. produced hæmolysis in 4 hours. These figures bear out the observation previously recorded, that the number of positive results obtained after 2½ hours' incubation is about one-third of the true number of hæmolytic specimens.

(2) Of the 128 specimens which proved to be hæmolytic 28 per cent. had a hæmolytic titre of 0·1 c.c. or less, 12 per cent. had a titre of 0·2 c.c., 14 per cent. a titre of 0·3 c.c., while the remaining specimens had a titre of 0·4 c.c. Specimens, 0·4 c.c. of which did not produce hæmolysis, were considered non-hæmolytic.

(3) Thirty-six per cent. of the 200 specimens were non-hæmolytic.

Several points may be noted:

(1) Since the hæmolytic activity of the urine excreted from day to day

varies, at times there being no hæmolytic power detectable, while at other times great activity is displayed, we may safely assume that had the 72 negative specimens been examined on subsequent days, the majority would have shown a hæmolytic activity. This, unfortunately, was not practicable.

(2) The difference between these results and those published in the previous paper is intelligible when it is remembered that the number of cases then examined was not so large, and that, though the cases were normal, they were selected because of the occurrence of hæmolytic substances; the estimation of the hæmolytic activity on many consecutive days in each of these cases would tend to give a higher percentage of positives than would be found in unselected normals.

(3) Since the percentage number of positives obtained by the examination of these 200 normals is distinctly less than the percentage number obtained from 300 cases of insanity, in which one estimation only was made (see paper referred to above), and since both these percentages are based on the examination of numbers of cases large enough for the results to be considered representative, it seems probable that there is a greater tendency to the excretion of hæmolytic substances in the urine among the insane, and doubtless also in many other pathological conditions, than there is in the normal individual. It is obvious, on the other hand, that the occurrence of such substances is not in itself abnormal, nor is it necessarily the accompaniment of a pathological condition.

CONCLUSIONS.

Sixty-four per cent. of normal unselected urines were found to be hæmolytic. If the non-hæmolytic specimens had been examined repeatedly they would probably have been found to be hæmolytic at some time. It seems that the presence of hæmolytic action is more frequent in insanity, but in itself is not abnormal.

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THE PHYSICO-CHEMICAL STATE OF SUGAR IN THE BLOOD.

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In spite of many and varied attempts, no decisive answer has yet been given to the question whether the glucose of the blood is present in the free crystalloid state, or whether it exists in the form of some colloidal complex. Henriques and Bing consider that most of it is in combination with lecithin as a complex resembling or identical with Drechsel's jecorin, and Kolisch, from clinical observations, holds the same view. Otto Loewi's experiments on pancreatic and phloridzin glycosurias led him to the similar conclusion that glucose was present normally in the form of a colloid complex, but that in the hyperglycæmia of pancreatic diabetes the excess was present in the crystalloid state, and thus was excreted at an increased rate when diuretics were given, just as urea and salts were. Lusk also believed that there were sugar-colloid combinations in normal blood.

Schenk, and later Rosenfeld, on the other hand, having demonstrated that the dialysability of glucose was not altered by the presence of protein or blood, regarded it as being present in the free state, though these conclusions have been criticised on theoretical grounds by Asher. Meyer contended that the free dialysability of the sugar was not incompatible with its normal existence in the blood in colloid form, unless the absence of enzymes capable of decomposing jecorin could be ensured. His search for such enzymes was, however, fruitless. Pflüger's arguments against the presence of free glucose appear to have been equally inconclusive, and the experiments of Michaelis and Rona, which showed some promise, were discountenanced by their later work.

It seems desirable that the problem, being of a physico-chemical nature, should be attacked by physico-chemical methods. At the suggestion of Prof. Kozawa use was made of an observation made by him in 1914.[1] He found that glucose can diffuse into human red corpuscles, with the result that their volume, as judged by the hæmatocrite volume of the blood, is increased. For this reason, as he showed in 1919[2], the red cells in diabetes mellitus are remarkably larger than the normal.

TECHNIQUE.

Osmotic experiments were made after the method described by Kozawa, which is briefly as follows:

He employed as the osmotic membrane human red corpuscles, which were mixed with the various experimental fluids, isotonic glucose solution (5·6 per cent.), normal serum, or the serum of a glycosuric patient having an excess of sugar. The volume of the red corpuscles thus treated was measured by the hematocrite. The sugar content was estimated by MacLean's modification of Bertrand's method.(3)

RESULTS.

Results with Human Red Corpuscles.

(1) The results of hematocrite examinations carried out by mixing equal parts of human red corpuscles (washed several times with sterilised physiological saline) and normal human serum by centrifugalising in a highly efficacious electric centrifuge, showed that there was no difference in the size of the red corpuscles. (The figures in the following tables are the hematocrite indices.)

TABLE I.

Sugar content.	Immediately.	1 hour.	2 hours.
0·102 per cent.	37	37	37
0·098 „	37	37	37

(2) My experiments also prove that glucose penetrates into human red corpuscles in isotonic glucose solution and increases the bulk of the cells, as is shown in the following table:

TABLE II.

Immediately.	½ hour.	1 hour.	1½ hours.	2 hours.	3 hours.	24 hours.
46	48·5	49	52·5	53	53·5	57

(3) The results of the experiments made with an isotonic saline-glucose solution and normal human red corpuscles were as follows, being similar to those of Table II:

TABLE III.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·100 per cent.	41	41	41	41	—
0·280 „	42	42	42	42	—
1·000 „	43	43·5	43·5	44	44
2·000 „	47	48	48	49	49
2·800 „	48	54	58	59	—

(4) The hematocrite indices of normal red corpuscles treated with normal human serum with the addition of isotonic glucose solution are shown in the following table. It will be seen that there is no difference in the diffusion whether the solvent be serum or isotonic saline glucose solution.

TABLE IV.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·776 per cent.	43	43	43	—	—
1·469 „	49	52	52	54	54

(5) The hematocrite indices of normal human red corpuscles in the serum of the diabetic patient (having an excess of sugar content) were as follows: They are practically the same as those obtained by the use of either isotonic saline-glucose solution or serum with the addition of isotonic glucose solution. By this evidence it is clear that there is no difference between the behaviour of sugar in the blood and that of free sugar as regards diffusion into the red corpuscle. In other words it may be concluded that sugar in the circulating blood shows the same properties as regards diffusion through the plasma-membrane of the human red corpuscles as free glucose does.

TABLE V.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·776 per cent.	43	43	43	—	—
1·035 „	48	49	51	53	53

CONCLUSION.

(1) As shown by Kozawa, glucose causes swelling of human red corpuscles But practically the same hematocrite results were obtained with human red corpuscles treated with the serum of a diabetic patient, normal serum with the addition of isotonic glucose solution, or isotonic glucose-saline solution.

(2) It may therefore be assumed that sugar in the circulating blood is present in the same physico-chemical character as in the solution of free glucose. In other words, it must be present in the circulating blood in the form of free sugar.

I wish to express my sincere thanks to Prof. Kozawa, who suggested this work to me and helped me throughout the course of the experiments.

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GROUPING OF INFLUENZA BACILLI.

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EITHER for preparing vaccine or for immunisation, the first requisite is to select suitable strains of influenza bacilli. I began, therefore, at the commencement of my experimental immunisation, to study to what extent strains varied as soon as it was concluded that the last influenza pandemic was caused by Pfeiffer's bacilli. The results of my work, with reference to vaccine and immune serum (1) were published in Japan in March, 1919. As reports on the varieties of influenza bacilli are now under discussion, a part of my experimental study dealing with various strains may be summarised here again. It is of interest not only as a problem in immunity but also in connection with the ætiology of the influenza pandemic.

Even at the time of the discovery of the influenza bacillus a suspicious strain was isolated from a child with broncho-pneumonia following diphtheria by Pfeiffer (2) himself, which he, being unable to differentiate distinctly from the typical strains by morphology, designated *Bacillus pseudo-influenza*. From time to time the "pseudo-influenza bacilli" have been isolated by various authors both from healthy or unhealthy respiratory tracts. Some were very similar in form to the typical influenza bacilli, but were non-pathogenic. Thus the very interesting question as to which was the real pathogenic germ of epidemic influenza remained unsettled up to the time of the last pandemic.

Before this last pandemic Grassberger (3) and Wollstein (4) reported varieties in the form of influenza bacilli, the last also showing immunity differences between different strains. More recently, independently of my study, Edwin O. Jordan (5) reported that ten strains produced indol while three did not. So far as differences in immunity reactions are concerned, Parker (6), Valentine and Cooper (7), Fleming and Clemenger (8), Small and Dixon (9) and Howard H. Bell (10) studied agglutination with a certain number of strains and their respective immune sera for different purposes. Some of them intended from the first to divide the strains into groups. No one, however, could divide the strains into certain definite groups, but each saw great differences among the strains. J. V. Cooke (11), who tried complement-fixation with sixteen strains, detected "no definite antigenetic relationship."

From my experience the morphological difference is not fixed enough to divide the strains into groups. Agglutination also yields no reliable results with influenza bacilli, as they are liable to spontaneous agglutination. Phagocytosis is too sensitive. Hence among the immune reactions complement-fixation seems most stable, even though the antigens are apt to be easily destroyed as shown below.

THE DIFFERENCE IN INDOL FORMATION.

The absolute distinction between strains of influenza bacilli is in the property of indol formation. Jordan was the first to show the production of indol by certain strains. He reported in June, 1919, that in his specially designed culture medium ten out of thirteen strains produced indol. In January, 1919, when it was important that certain strains should be chosen for the purpose of making vaccine and immune serum, I obtained proof of indol formation in the following way: The ordinary slightly alkaline bouillon was heated over 90° C. in a Koch's steriliser, taken out, and defibrinated horse blood added in a 10 per cent. proportion. It was then thoroughly shaken to dissolve the blood-corpuscles. When it changed to a dark brown mixture it was filtered, first through filter-paper and then through Berkefeld's clay-filter. The final filtrate was collected in a sterilised flask and then divided into sterilised test-tubes, 10 c.c. for each. To test its sterility it was kept at 37° C. for a day. If sterile, it remains quite clear, tinged a little yellow. A platinum loop of culture of influenza bacilli was then inoculated into it. The strains inoculated were isolated from the expectoration of typical influenza cases, simple or complicated. The indol reaction was tested after the Kitasato-Salkowsky method.

TABLE I.—*Indol Formation by New and Old Strains in Four Days' Growth.*

The new strains had been isolated for two months, and the old strains had been isolated fifteen to eighteen months before: *n* for the new, *o* for the old strains.

Number of strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
New or old	n	n	n	n	n	n	o	o	o	o	o	o	o	o	o
Indol in four days' growth	−	+	+	+	+	−	+	+	+	−	−	+	−	−	+

The table shows two negative strains out of six new strains, and four negative ones out of nine old. It seems most probable that subculture (made every fourth day) has no influence upon the property of indol formation.

TABLE II.—*Indol Formation by several Strains in Twelve Days' Growth.*

Number of strain	16	17	18	19	20	21	22	23	24	25	26	27	28	29	1	7
Indol in twelve days' growth	+	+	−	+	+	−	−	+	+	−	+	−	+	+	−	+

Among 29 old and new strains tested there are 18 positive indol strains (62·0 per cent. of the total) and 11 negative indol (38·0 per cent. of the total

number of strains). Some strains seemed to produce indol a little quicker than others, owing probably to the rapidity of their growth, but in full growth there was no noticeable difference. When I referred to patients' records no relation was found between the production of indol and the severity of disease.

Hence the influenza bacilli were definitely differentiated into two characteristic groups—one positive indol, the other negative indol.

THE DIFFERENCE IN THE VIRULENCE AND TOXICITY OF THE TWO GROUPS.

Wollstein reported a difference between the virulence of a strain from blood in a case of meningitis and of another from the respiratory tract in bronchitis. The virulence was strong in the strain from meningitis—a 1 kgrm. rabbit being killed in 18 to 30 hours after the inoculation of an agar slant culture, while that from the respiratory tract was avirulent. In examining the virulence of influenza bacilli the length of time they have been maintained in artificial culture should be taken into account. In my strains the newly isolated cultures from typical influenza had usually a strong virulence, killing in a day a 10 grm. mouse with 0·1 to 0·2 mgrm. culture, or a 200 grm. guinea-pig with 1·0 to 2·0 mgrm. when injected into the peritoneal cavity. On the other hand, the virulence of strains from carriers proved very weak, but strengthened with the onset of the fever. For instance, a new culture, isolated in November, 1918, from my laboratory girl when she was a carrier, did not kill a 10 grm. mouse with even 2 mgrm., while the first culture when isolated on January 4th, 1919, during the attack of fever killed a mouse of the same weight with 0·05 mgrm.

All strains, however, lost a great deal of their original virulence with each subculture and finally became avirulent. As the decrease of virulence on subculture is not the same with each strain, it is unreasonable to compare the virulence of some strains with that of others, either in the same or in different generations of the culture, except in the first generation. Summed up, there was no remarkable difference of virulence between the positive indol strains and the negative, as determined by the minimal lethal dose of the first culture.

In order to determine the toxicity sixteen strains were tested, eight strains being positive indol and the others negative. The eighteen hours' growth of each strain on heated blood-agar was emulsified separately strain by strain in 0·5 per cent. carbolic saline at the rate of 1·0 mgrm. in 1·0 c.c. After standing the emulsions for two hours in the incubator at 37° C. separate strains were injected into rabbits intravenously. It was proved by cultivating that the bacilli in the emulsion were killed at the time of injection, hence there was no time for the toxicity to diminish by the decomposition of the killed bacilli. The quantity injected was 1 c.c. containing 1 mgrm., or when diluted 0·5 mgrm. killed bacilli. The injected rabbits which died were weighed and an autopsy made occasionally. They were observed for ten days after the injection. Table III gives the result of this experiment.

The susceptibility of rabbits to the bacillary toxin is somewhat variable, but it seems that the toxicity is a little stronger in negative indol strains than in positive. The weight of the animals is very much diminished in the case

TABLE III.—*The Toxicity of Strains.*

Rabbits.	Weight before injection.	Number of strain.	Indol formation.	Quantity injected		Survived or succumbed.	Post-mortem examination.	
				Actual	Per kgrm.		Weight.	Special changes.
I	2355 grm.	24	+	1 c.c. = 1·0 mgrm.	0·42 mgrm.	†3*	2115 grm.	General anæmia, spotted bleeding in lungs.
II	1970 „	4	+	Ditto.	0·50 „	†4	1800 „	General anæmia, spotted bleeding in lungs, diarrhœa.
III	2090 „	28	+	„	0·49 „	—	—	—
IV	2235 „	7	+	„	0·44 „	—	—	—
V	2175 „	23	+	„	0·46 „	—	—	—
VI	2920 „	12	+	1 c.c. = 0·5 mgrm.	0·17 „	†1	—	—
VII	2175 „	15	+	Ditto.	0·23 „	—	—	—
VIII	2750 „	9	+	„	0·18 „	—	—	—
IX	2950 „	12	+	„	0·17 „	—	—	—
X	2380 „	27	−	1 c.c. = 1·0 mgrm.	0·42 „	—	—	—
XI	2755 „	25	−	Ditto.	0·36 „	†5	1945 grm.	General anæmia, bleeding in lungs and pericardium.
XII	2175 „	22	−	„	0·46 „	†1	1905 „	Hyperæmia in colon, diarrhœa, spotted bleeding in lungs.
XIII	2650 „	11	−	„	0·38 „	†1	2630 „	Bleeding in lungs, ovary and supra-renal glands.
XIV	1975 „	10	−	„	0·50 „	†3	1530 „	Hyperæmia and bleeding in lungs.
XV	2500 „	1	−	1 c.c. = 0·5 mgrm.	0·20 „	—	—	—
XVI	2620 „	6	−	Ditto.	0·20 „	—	—	—
XVII	2220 „	13	−	„	0·23 „	—	—	—

* †1 or †2 means death one day or two days after the injection.

of death a few days after the injection. Diarrhœa usually occurs in acute intoxications. The animals which survived the injection for a few days had marked hæmorrhages.

CROSS COMPLEMENT-FIXATION WITH ANTIGENS AND IMMUNE SERA OF SEVERAL STRAINS OF THE TWO GROUPS.

According to my experiments complement-fixation seems to be the most stable and reliable immune reaction. Agglutination is not reliable as the bacilli are so liable to undergo spontaneous agglutination, and it was found impossible to prevent this. Complement-fixation was therefore chosen both for the standardisation of immune serum or for the classification of influenza bacilli.

Six strains were used to obtain their respective immune sera, three being positive indol and the other three negative indol. Rabbits were intravenously injected separately, strain by strain. Each was twice injected with 0·5 mgrm. bacilli killed by carbolic acid, and at the period of the highest immunity they were bled. No disinfectant for conservation was added to the sera which were tested a few days after. Before testing each serum was heated at 57° C. for half an hour.

Antigen was made as follows: The 48 hours' growth of each strain on heated blood agar was separately emulsified in 0·5 per cent. carbolic-saline in the proportion of 2 mgrm. in 1 c.c. fluid. This emulsion was kept at 37° C. overnight so that the bacilli were killed and moderately autolysed. It is most important that there should be a certain degree of autolysis in the antigen that it may be effective. The antigen is killed in an incubator rather than at a higher temperature, because too much autolysis may occur and the antigen become ineffective.

The hæmolytic system was sheep's red corpuscles (5 per cent. suspension of blood-corpuscles) with a rabbit *versus* sheep's immune body.

As complement guinea-pig's fresh serum was used. For use it was preliminarily tested, so that the quantity employed was 0·5 c.c. diluted fourteen times—that is, double units for this system. Next the antigens were tested for their anti-complementary effect in this system and also for their antigenic power with regard to the antibody.

TABLE IV.—*The Anti-complementary Effect of each Antigen.*

Each tube contains 0·5 c.c. antigen; its corresponding weight of bacilli is shown in figures: H = complete hæmolysis; **D** = complete deviation; D = moderate deviation; d = slight deviation.

Number of strain.	Quantity of antigen in each strain (mgrm.).			
	0·5	0·25	0·12	0·06
12	H	H	H	H
13	H	H	H	H
1	d	H	H	H
15	H	H	H	H
6	**D**	d	H	H

TABLE VI.—*Showing the Antigenic Effect of 0·12 mgrm. of each Strain of Influenza Bacilli on its own as well as on Immune Sera of the Other Strains, cross-examined as shown. The immune serum is diluted so that each tube contains 0·5 c.c. To the first tube of the first antigen tested in each series saline was added instead of antigen as a control of the anti-complementary effect of the serum.*

The quantity of serum in the columns numbered 1 to 6 are: No. 1, 0·05 c.c.; No. 2, 0·025 c.c.; No. 3, 0·012 c.c.; No. 4, 0·006 c.c.; No. 5, 0·003 c.c.; No. 6, 0·0015 c.c.

With the immune serum of No. 13 (positive indol strain).

Indol.	Antigen No.	Quantity of the immune serum. 1	2	3	4	5	6
−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	D	H
−	1	**D**	**D**	**D**	D	H	H
−	13	**D**	**D**	**D**	**D**	D	d
+	12	D	H	H	H	H	H
+	9	**D**	**D**	**D**	D	d	H

With the immune serum of No. 6 (negative indol strain).

−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	**D**	d
−	1	**D**	**D**	**D**	**D**	D	H
−	13	**D**	**D**	**D**	**D**	d	H
+	12	**D**	D	d	H	H	H
+	9	**D**	**D**	D	d	H	H

With the immune serum of No. 18 (negative indol strain).

−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	D	H	H
−	1	**D**	**D**	**D**	D	H	H
−	13	**D**	**D**	**D**	D	H	H
+	12	H	H	H	H	H	H
+	9	d	H	H	H	H	H

With the immune serum of No. 1 (negative indol strain).

−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	d	H
−	1	**D**	**D**	**D**	**D**	**D**	**D**
−	13	**D**	**D**	**D**	**D**	D	H
+	12	**D**	D	H	H	H	H
+	9	**D**	D	H	H	H	H

With the immune serum of No. 15 (positive indol strain).

−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	D	H
−	1	**D**	**D**	**D**	D	H	H

With the immune serum of "H" strain (indol formation was not examined).

−	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	**D**	**D**
−	1	**D**	**D**	**D**	D	d	H
−	13	**D**	**D**	**D**	**D**	**D**	D
+	12	**D**	**D**	D	d	H	H
+	9	**D**	**D**	**D**	**D**	d	H

TABLE V.—*Showing the Antigenic Effect on the Serum of a Rabbit which was Strongly Immunised with Strain No. 12.*

The volume of antigen in each tube is 0·5 c.c.; the weight of bacilli contained corresponds to the figures shown. To each tube 0·5 c.c. of the immune serum six times diluted was added.

Number of strain.	Quantity of antigen in each strain (mgrm.).					
	0·5	0·25	0·12	0·06	0·03	0·015
12	**D**	**D**	**D**	d	H	H
13	**D**	**D**	**D**	**D**	d	H
9	**D**	**D**	**D**	**D**	d	H
1	**D**	**D**	**D**	d	H	H
15	**D**	**D**	**D**	D	d	H
6	**D**	**D**	**D**	**D**	D	d

From these two preliminary tests 0·12 mgrm. of each antigen was found to be the optimum amount—that is, 0·5 c.c. four times diluted—and this quantity was then cross-tested with various immune sera as shown in Table VI.

As the table shows, no definitive antigenic relationship could be detected between the positive and negative indol groups, nor among any of the strains examined. Nevertheless some yield a much more potent antigen than others even when prepared in the same way, and this may account for the fact that a particular antigen does not necessarily react most strongly with its respective immune serum. Thus the varying effect of each antigen may be due mainly to the properties of each strain. Strains No. 6 and No. 9 were very liable to yield to spontaneous agglutination and seemed to be closely allied. Their antigenic effects, however, were quite different, the former being very strong, the latter weak.

CONCLUSIONS.

(1) Influenza bacilli can be divided definitely into two groups according to the character of indol formation. Out of 29 strains examined, 18 strains (62 per cent.) form indol. No intermediate strains are found. It is very probable that this character of indol formation is not alterable by prolonged subculture.

(2) By morphological and immunological study it is impossible to divide influenza bacilli into groups, because there are found as many intermediate strains as there are strains examined.

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